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Partial inhibition of AT-EAE by an antibody to ICAM-1: clinico-histological and MRI studies

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Abstract

The role of quantitative proton magnetic resonance imaging (MRI) for the evaluation of immunopathological lesions in the CNS was studied in adoptively transferred experimental allergic encephalomyelitis (AT-EAE). We utilized a recently established treatment model, inhibition of the cell adhesion molecule ICAM-1 by the monoclonal antibody 1A-29. The animals were scanned on days 3, 5 and 7 after injection of encephalitogenic T-cells, before and after bolus injection of Gd-DTPA by performing $T1$-measurements to assess the integrity of the blood-brain barrier (BBB). On day 7, immunohistochemistry was performed looking for T-cells, activated macrophages, and albumin staining. There was clinical evidence of partial inhibition of AT-EAE in rats treated with antibodies against ICAM-1. This finding was in line with a significantly reduced number of T-cells in the medulla. However, the number of activated macrophages and the distribution of albumin did not differ from untreated AT-EAE animals. The histological findings are in agreement with the MRI data before and after Gd-DTPA injection which were similar in treated and untreated AT-EAE rats on day 3 and 5. On day 7 after Gd-DTPA injection there was evidence of a delayed breakdown of the BBB in the treated rats. The observation of a dissociation of clinical and MRI findings, especially evidence of Gd-enhancement despite clinical improvement, may be important in the context of interpreting MRI studies in MS patients in treatment trials.

Keywords: Multiple sclerosis; Experimental allergic encephalomyelitis; Magnetic resonance imaging; Blood-brain barrier; Adhesion molecules

1. Introduction

The intercellular adhesion molecule-1 (ICAM-1) is an early marker of immune activation and is important for the migration of primed T-cells from the blood into inflamed tissue (Dougherty et al., 1988; Springer, 1990; Oppenheimer-Marks et al., 1991; Shimizu et al., 1991, 1992). Serum levels of soluble ICAM-1 are elevated in multiple sclerosis (MS) patients with active disease as evidenced either clinically or by Gd-DTPA enhanced MS-lesions on brain magnetic resonance imaging (MRI) (Hartung et al., 1993; Sharief et al., 1993). We reported successful treatment of actively induced experimental allergic encephalomyelitis (EAE) in Lewis rats with a monoclonal antibody (mAb) against ICAM-1 (α-ICAM-1 mAb) (Archelos et al., 1993). More recently others showed similar findings in active EAE in guinea pigs using an antibody against α-4 integrin (Kent et al., 1995). However, there are conflicting data on the therapeutic role of α-ICAM-1 mAb in adoptively transferred (AT)–EAE. While we could not demonstrate a significant effect of α-ICAM-1 mAb in AT-EAE induced in Lewis rats (Archelos et al., 1993), Baron et al. (1993) using a murine model found that high dose α-ICAM-1-mAb treatment ameliorated the clinical course of the disease.

In this study, we repeated our earlier experiments on AT-EAE with a higher dose of the mAb. We extended assessment of the effects of α-ICAM-1-mAb antibodies from clinical observation to serial MRI and immunohistochemical analysis.

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2. Materials and methods

2.1. Animals

Female Lewis rats, 6 to 8 weeks old with a body weight of 150–180 g, were obtained from the Zentralinstitut für Versuchstierzucht Hannover and Charles River, Sulzfeld (Germany). All experiments were performed with approval by Bavarian State Authorities.

2.2. Induction of adoptive transfer (AT)–EAE

AT-EAE was induced by injection of 9×10^6 MBP-specific, CD^+ activated T-cells into the tail vein. The encephalitogenic T-cells were generated and maintained as described elsewhere (Jung et al., 1992). In brief, T-cells (3×10^5/ml) were restimulated with irradiated (3000 rad) thymocytes ($1{,}5 \times 10^7$/ml) as antigen presenting cells (APCs) and MBP (20 μg/ml). 72 h later activated T-cell blasts were separated from cell debris by centrifugation on Ficoll (Nycomed AS, Oslo, Norway) gradients at 4°C, washed twice and 9×10^6 blasts were injected into the tail vein in 1 ml Dulbecco's modified Eagle's medium (Gibco, Eggenstein, Germany). A total of 18 rats injected with the MBP-specific T-cell line. Twelve rats i.v. injected with medium alone served as normal controls.

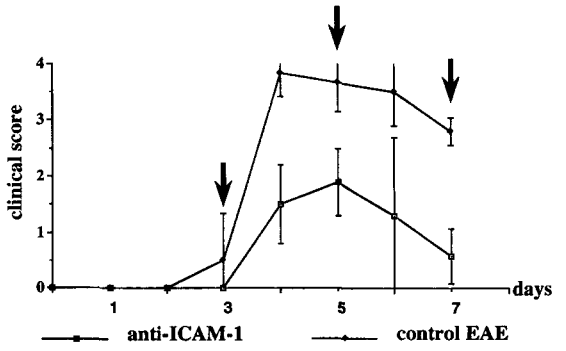

Fig. 1. Time course of clinical disease of α-ICAM-1-mAb treated and untreated AT-EAE animals. Arrows denote days of MRI investigation. Between days 4 and 7 the differences in clinical scores were statistically significant ($p < 0.001$). Bars indicate standard deviation.

2.3. Antibody to ICAM-1 and isotype control monoclonal antibody

The monoclonal antibody of IgG1 isotype (clone 1A-29) directed to ICAM-1 (Tamatani and Miyasaka, 1990) and

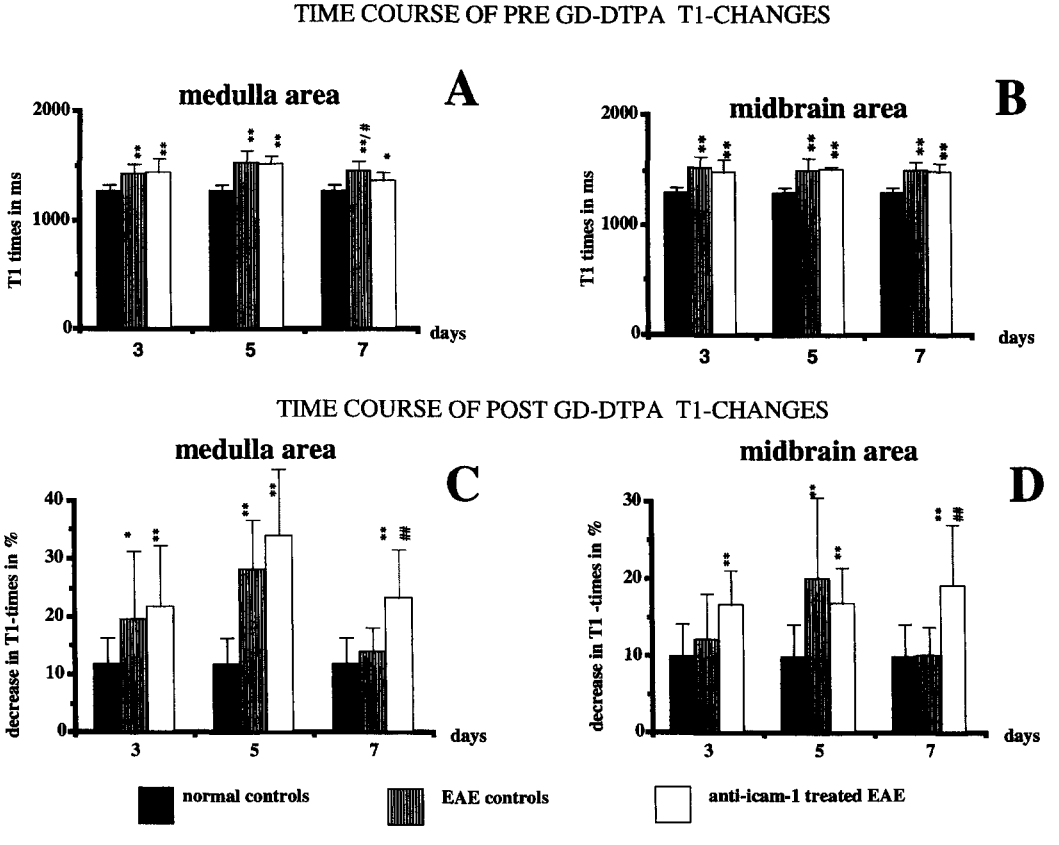

Fig. 2. Anti-ICAM-1-mAb treated and PBS treated AT-EAE: On days 3, 5, and 7 after transfer of encephalitogenic T-cell $T1$-times prior to Gd-DTPA injection (A,B) and $T1$-times post Gd-DTPA injection (C,D) (decrease in percent: $T1_{\text{pre-GD-DTPA}} - T1_{\text{post-Gd-DTPA}}/T1_{\text{pre-Gd-DTPA}}$) in the medulla and midbrain. Statistical comparison between normal rats and both AT-EAE groups (treated or untreated): $^* = p < 0.05$, $^{**} = p < 0.01$; and between treated and untreated AT-EAE rats: $\# = p < 0.05$; $\#\# = p < 0.01$.

the control IgG1-antibody specific of the CD2 ligand on sheep red blood cells (clone L180) (Hünig et al., 1986) were prepared from culture supernatants of the hybridoma cells as described elsewhere (Archelos et al., 1993).

2.4. Treatment and clinical scoring

The α-ICAM-1-mAb treated group ($n = 6$) received 5 mg of 1A-29 in a volume of 0.5 ml PBS daily, from day 0 to day 6, either by intracardiac or intravenous injection. If MRI was performed, the animals received 1A-29 after finishing the MRI acquisition. Control EAE rats ($n = 6$) received the same volume of PBS. In a separate MRI-control group normal rats ($n = 3$) received α-ICAM-1 mAb (5 mg of 1A-29) using the same treatment protocol as for the EAE α-ICAM-1-mAb treated group, and similarly AT-EAE rats ($n = 3$) were daily injected for seven consecutive days, starting from day 0, with 5 mg of an isotype control mAb.

The severity of the disease was assessed using a scale from 0 to 6. Scoring was performed by a masked observer as follows: 0 = normal; 1 = weakness of tail tip, 2 = paralysis of tail, 3 = mild to moderate paraparesis, unsteady gait; 4 = severe paraparesis; 5 = tetraplegia; 6 = moribund and death.

2.5. Magnetic resonance imaging (MRI)

In vivo MRI was performed on a Bruker Biospec NMR system (7 Tesla, 200 mm horizontal bore). Anesthesia for MRI was induced with 5% isoflurane and then maintained at 1% in oxygen flowing at a rate of 1.5 l/min. For $T1$-mapping we used a snapshot FLASH sequence as described elsewhere (Haase, 1990; Deichmann and Haase, 1992; Nekolla et al., 1992) In short, $T1$-calculations were performed on the basis of 16 snapshot FLASH images acquired within 2.5 s after an inversion pulse. Using 64 phase steps with an echo time (TE) of 1.4 ms and a repetition time (TR) of 2.7 ms, each image acquisition took 170 ms. Eight averages were taken for each image, using an interval of 14 s between two inversion pulses to improve on signal to noise ratio. $T1$-maps were performed in each animal before and after a bolus injection of Gd-DTPA via an indwelling venous catheter (0.8 mmol/kg GD-DTPA). Eight transaxial slices through the brain and brain stem were obtained from each animal. An MRI adapted stereotactic apparatus was used to ensure exact

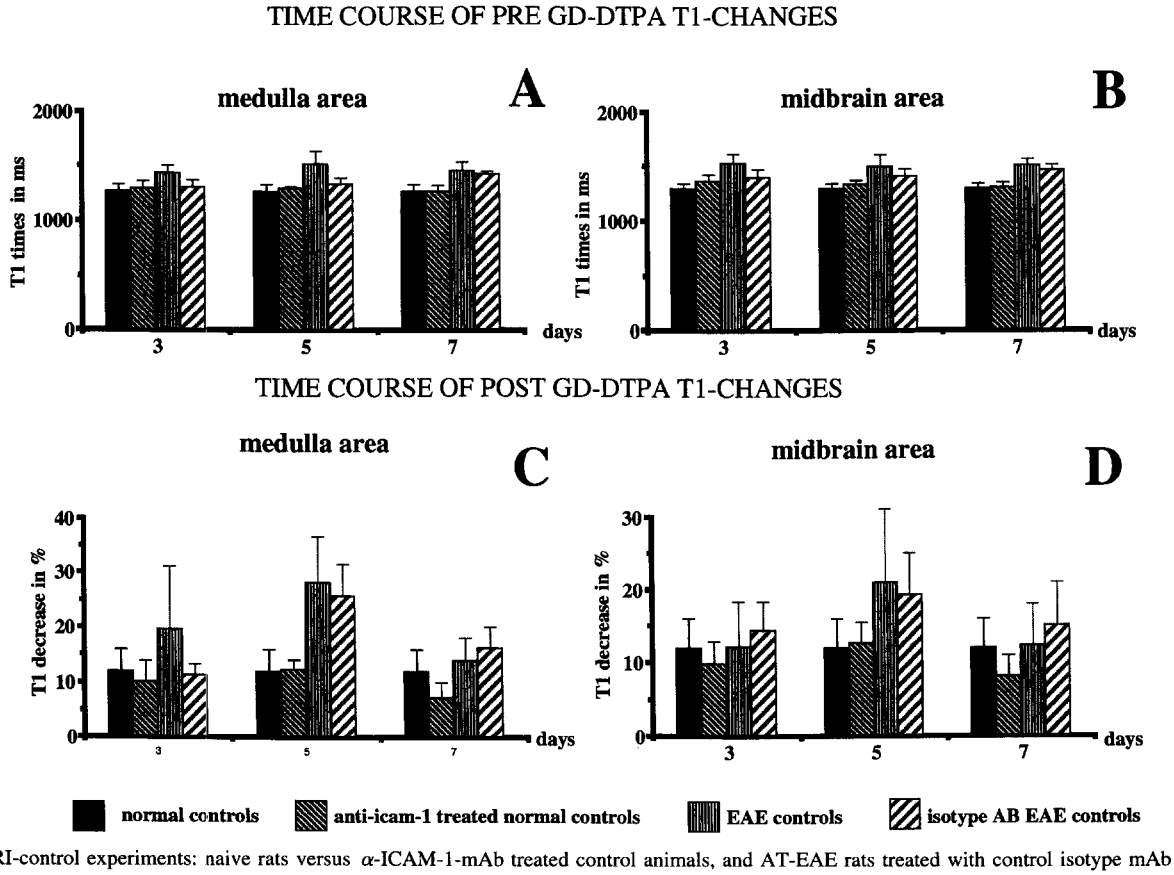

Fig. 3. MRI-control experiments: naive rats versus α-ICAM-1-mAb treated control animals, and AT-EAE rats treated with control isotype mAb or PBS: On days 3, 5, and 7 after transfer of encephalitogenic T-cell $T1$-times prior to Gd-DTPA injection (A,B) and $T1$-times post Gd-DTPA injection (C,D) (decrease in percent: $T1_{\text{pre-GD-DTPA}} - T1_{\text{post-Gd-DTPA}}/T1_{\text{pre-Gd-DTPA}}$) in the medulla and midbrain.

repositioning. Scanning was performed on days 3, 5, and 7 post-cell injection (pi).

2.6. Histology

On day 7 within 1 to 4 h after MRI acquisition rats were anaesthetized with sodium pentobarbital (Narcoren®, Rhône Merieux, Laupheim, Germany) and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffered saline, pH 7.4. Brains were post-fixed for 3 h in the same fixative. According to a stereotactical atlas (Pelligrino et al., 1979) the brain was cut transversely at the level of the tentorium and parallel to it, so that dorsally the colliculi inferiors and ventrally the entry of the trigeminal nerves were in the same plane. For embedding in paraffin, the brains were cut in approximately 3 mm thick transverse slices, and sections were collected from the midbrain level. 10 μm thick sections were cut from caudal to rostral, approaching the level of the colliculi superiors dorsally and the pons ventrally. Immunohistochemistry was performed by the ABC-method using the following anti-rat antibodies: anti-pan T-cell (B115-1, Holland Biotechnology, Leiden, The Netherlands), 1:500; ED1 for staining of macrophages and microglia (Serotec, Camon, Wiesbaden, Germany), 1:500; anti-rat-albumin (Nordic, Bochum, Germany), 1:100. Slides for albumin staining were pretreated with hydroxylamine (0.9% in PBS, 30 min). Sections were incubated with 10% heat-inactivated porcine serum to block unspecific protein binding. MAb's were diluted in 0.05 M Tris/2% NaCl (B115-1; ED1) or 0.05 PBS (α-albumin) containing 1% porcine serum and preincubated for 1 or 2 h, respectively before allotment on the slides. After treating the sections with 0.5% H_2O_2 in methanol to inhibit endogenous peroxidase activity they were incubated with a biotinylated anti-mouse-Ig antibody (Dakopatts, Hamburg, Germany) for 45 min, which had been preincubated with the same volume of Lewis rat serum for 15 min at 37°C. The avidin–biotin–peroxidase complex (ABC) was allowed to react for 30 min. The peroxidase labelling was visualized by incubation with a solution containing 0.05% 3,3'-diaminobenzidine tetrahydrochloride and 0.05% H_2O_2. All sections were counterstained with haematoxylin for 10 s. For quantitative comparison staining of the sections of all experimental animals was performed at one time point for each antibody.

Preincubation of the anti-rat-albumin antibody with rat albumin 100 mg/ml (Sigma) abolished the staining on brain sections demonstrating the specificity of the mAb.

2.7. Quantification

In 10 μm paraffin sections of each experimental animal all labelled parenchymal T-cells, or ED1 positive cells

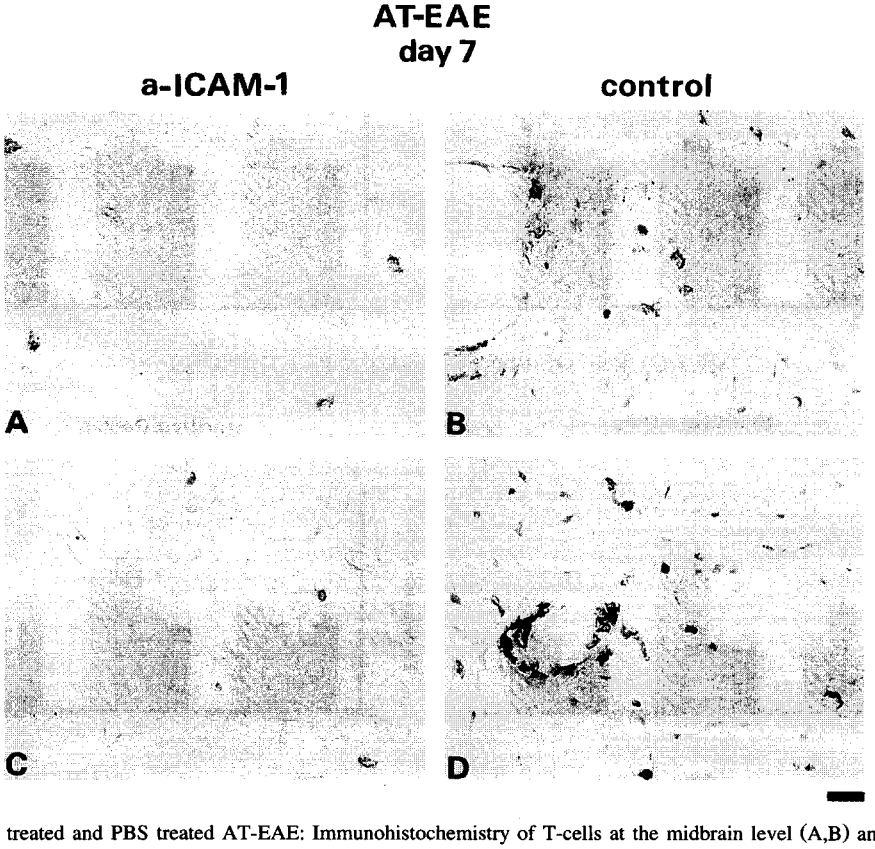

Fig. 4. Anti-ICAM-1-mAb treated and PBS treated AT-EAE: Immunohistochemistry of T-cells at the midbrain level (A,B) and in the medulla (C,D) of α-ICAM-1-mAb treated (A,C) and control EAE rats (B,D) on day 7 of AT-EAE. In the treated animals only a few T-cells were present (A,C) as compared to controls (B,D). Bar = 20 μm.

were counted. For each group the mean value and standard deviation was determined. Only ED1 positive and nucleated structures were counted to exclude overestimation of cell numbers due to ED1 staining of cell processes. Albumin staining was quantified in two sections of each animal using a grading system from 0 to 4 and the mean values were determined. Albumin scores were given according to the staining pattern around the blood vessels and in surrounding tissue. Grade 0: no positive staining could be detected at vessels; grade 1: a thin rim of staining in the endothelial layer and at its abluminal surface; grade 2: a thicker ring of reaction product from the endothelial layer to the area around the vessels; grade 3: diffuse halo of peroxidase product around most of the vessels resulting from diffusion of albumin into the parenchyma; grade 4: a more or less homogeneous staining in rostral and dorsal parts of the slides suggests a diffusion of albumin within the whole section. Usually, albumin staining was weak in the rostral as compared to the caudal areas of the brain stem. Means of the grades in the areas were calculated and in addition, grand means of the two adjacent sections.

2.8. Statistical analysis

For statistical analysis the Wilcoxon signed rank test was used in all calculations of significance between control, and treated and untreated disease groups.

3. Results

3.1. Clinical findings

Both treated and untreated EAE animals developed clinical signs of disease, but α-ICAM-1-mAb treated animals were significantly less affected than untreated AT-EAE animals (Fig. 1).

3.2. MRI findings

3.2.1. Before injection of Gd-DTPA

Before injection of Gd-DTPA there was in both untreated and treated EAE animals a significant increase in $T1$-times in the medulla and midbrain area in comparison to normal control rats. In the α-ICAM-1-mAb treated group, on day 7, pre-Gd-DTPA $T1$-times were shorter compared to the untreated EAE animals in the medulla area. The latter difference even if small reached statistical significance (Fig. 2A,B). Normal control rats injected with α-ICAM-1 mAb showed no difference in pre-Gd-DTPA $T1$-relaxation times compared to normal rats. With regard to pre-Gd-DTPA $T1$-relaxation times AT-EAE animals injected with a control isotype antibody did not differ from AT-EAE rats injected with saline (Fig. 3A,B).

3.2.2. After injection of Gd-DTPA

As expected from previous studies (Seeldrayers et al., 1993; Morrissey et al., 1996), after injection of Gd-DTPA we observed in untreated EAE rats a significant decrease in $T1$-times in the medulla on days 3 and 5, and in the midbrain on day 5 compared to normal controls. However, in contrast to the changes in $T1$-times prior to injection of Gd-DTPA, in the α-ICAM-1-mAb treated EAE rats the $T1$-decrease after Gd-DTPA injection tended to be greater than in the untreated EAE rats in the medulla and midbrain on day 3 and 5. On day 7 in both areas this finding was significant (Fig. 2C,D). Normal control rats injected with α-ICAM-1 mAb showed no difference in post-Gd-DTPA $T1$-relaxation times compared to normal rats. With regard to post-Gd-DTPA $T1$-relaxation times in AT-EAE animals injected with a control isotype antibody values did not differ from AT-EAE rats injected with PBS (Fig. 3C,D).

3.3. Histology

Morphological analysis was performed on day 7 post-cell transfer. In sections of the midbrain most T-cells were located in the ventral part, i.e. near the entry of the

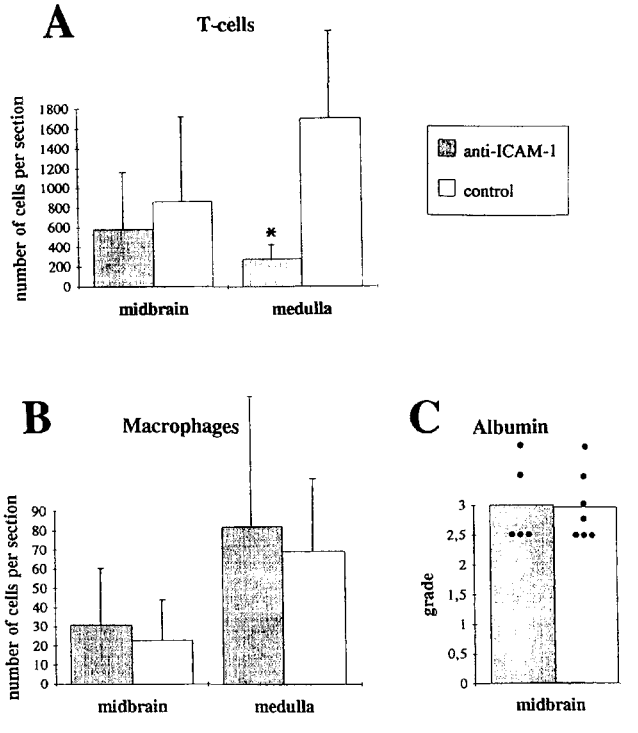

Fig. 5. (A) Comparison of the number of T-cells in the α-ICAM-1-mAb treated and untreated control group. In the midbrain and in the medulla the number of T-cells is higher in the untreated rats than in the α-ICAM-1-mAb treated group ($p < 0.05$ in the medulla). (B) and (C): Comparison of the number of macrophages and albumin deposits. There is no difference in the number of macrophages and in the albumin score between the two groups. Columns represent the mean values with the standard deviation in (A), and (B). In (C), the dots represent the individual score values of single experimental animals. Box areas represent mean and bars the standard deviation.

trigeminal nerve. In the medulla T-cells were distributed throughout the section (Fig. 4). In AT-EAE animals treated with α-ICAM-1 mAb the average number of T-cells was lower as compared to control EAE rats (Fig. 5A). For the medulla this difference reached statistical significance ($p < 0.05$), although the variability between animals was high (Fig. 5B).

Macrophages and microglial cells were assessed by labelling with the ED1-antibody (Lassmann et al., 1993; Damoiseaux et al., 1994). In the medulla, there was no significant difference in the numbers of ED1-labelled cells between the treated and untreated group (Fig. 5B).

The immunohistochemical staining of albumin served as a read-out system for oedema caused by the disruption of the blood-brain barrier (BBB). Quantification of the deposited albumin was performed as described above. There was no difference between the two groups (Fig. 5C). Most animals showed a staining pattern of grade 2 and 3 (resulting in a mean value of 2.5), which means that one part of the blood vessels was stained with a surrounding rim, the other part of the blood vessels with a widespread halo of peroxidase reaction product (Fig. 6).

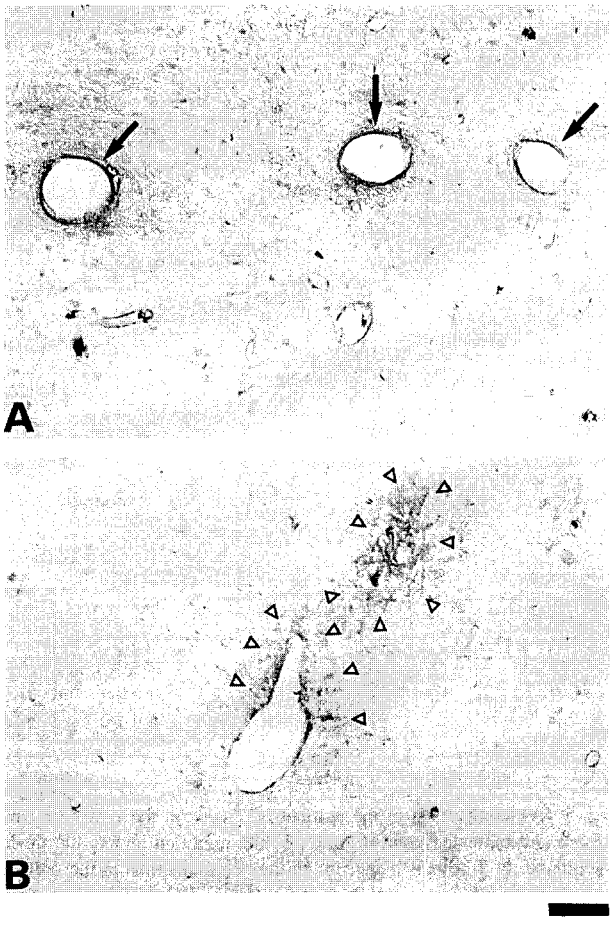

Fig. 6. Albumin immunoreactivity in paraffin sections showing the typical staining patterns of blood vessels (arrows). (A): ring of reaction product representing grade 2. (B): halo of positive immunoreactivity reaching into the parenchyma characteristic of grade 3 (arrowheads). Bar = 50 μm.

4. Discussion

In this study AT-EAE animals with and without treatment by α-ICAM-1 mAb were investigated by serial MRI at a time when BBB breakdown was seen with MRI using Gd-DTPA (Seeldrayers et al., 1993; Morrissey et al., 1996) and by immunohistochemistry. The clinical as well as the neuropathological data obtained in our present study suggest that α-ICAM-1-mAb therapy in AT-EAE ameliorates the inflammatory reaction in the central nervous system (CNS) and leads to a less severe disease. This is reflected by the significantly lower numbers of T-lymphocytes within the lesions 7 days after cell transfer. ED-1 positive macrophages may persist in EAE lesions for prolonged time periods (Lassmann et al., 1991, 1993) as seen in the present experiments. The view of a less severe CNS inflammation in α-ICAM-1-mAb treated animals is further corroborated by the clinical course of the disease and by the significant differences in pre-Gd-DTPA $T1$-changes suggestive of a more rapid resolution of brain oedema. Performing $T2$-weighted MRI the latter finding was also observed by Kent et al. in EAE animals using a monoclonal antibody against α-4 integrin (Kent et al., 1995).

This interpretation, however, is challenged by significantly increased Gd-DTPA leakage found at 7 days after transfer in α-ICAM-1-mAb treated animals. Thus, it has to be considered that α-ICAM-1-mAb treatment may directly affect the BBB-permeability. Archelos et al. (1993) observed that the α-ICAM-1 mAb also binds to the endothelia in spleens and may induce necrosis of these cells under certain conditions. By analogy, the BBB may be damaged by the mAb bound to brain endothelia which had upregulated their expression of ICAM due to the local inflammation (Cannella et al., 1991). An alternative explanation for this apparent discrepancy is the possibility that clinical disease severity in our rats is associated more closely with spinal cord inflammation (Pender et al., 1989; Chalk et al., 1994). This possibility does not, however, bear on the discussion as to the correlation of MR findings and histological signs of inflammation with oedema.

Gd-DTPA does not cross the BBB under normal circumstances and is therefore regarded as a marker of the integrity of the barrier (Grossmann et al., 1986; Gonzalez-Scarano et al., 1987; Kappos et al., 1988; Kermode et al., 1988; Miller et al., 1988, 1993; Bastianello et al., 1990; Harris et al., 1991; Thompson et al., 1991; Barkhof et al., 1992; Capra et al., 1992; McFarland et al., 1992). The difference in our pre- and post-Gd-findings is not unexpected considering the different molecular weight of Gd-DTPA (500 D) and albumin (65 kD). Indeed, in chronic EAE in guinea pigs different patterns of Gd-enhancement comparing Gd-DTPA and Gd-labelled albumin have been

observed (Hawkins et al., 1990). Another explanation could be selective transport of these components through the BBB. Gd-enhancement per se reflects active inflammation in the animal models of MS (Hawkins et al., 1990, 1991; Namer et al., 1992, 1993; Seeldrayers et al., 1993; Morrissey et al., 1996), and in MS patients (Nesbit et al., 1991; Katz et al., 1993), and therefore is regarded as an in vivo marker of disease activity. In vivo MRI studies in EAE models (Hawkins et al., 1990, 1991) have shown that Gd-enhancement is always associated with inflammatory infiltrates. Using a quantitative approach to assess the natural history of BBB breakdown in AT-EAE and applying an ultrafast NMR technique (Haase, 1990; Deichmann and Haase, 1992; Nekolla et al., 1992) our group recently demonstrated in AT-EAE rats that post-Gd-DTPA $T1$-values broadly parallel inflammatory infiltrates (Seeldrayers et al., 1993), and especially parallel to the time course of macrophage and microglia activation, and to a lesser degree the time course of T-cell infiltration (Morrissey et al., 1996). This is in agreement with our present MRI and immunohistochemical findings in α-ICAM-1-mAb treated AT-EAE rats where ED-1 staining was similar in the treated and control AT-EAE group, and similar changes of $T1$-times after Gd-injection were found between the two AT-EAE groups and the normal control animals.

Anti-ICAM-1-mAb treatment of EAE animals may act on several different levels. ICAM is involved in leucocyte homing in peripheral lymphatic tissue. Thus, α-ICAM-1-mAb therapy may interfere with proper lymphocyte traffic in general. These mechanisms are likely to be involved in the blockade of inflammation in EAE animals (Archelos et al., 1993). Moreover, α-ICAM therapy may directly act on the BBB. ICAM is upregulated on cerebral endothelial cells during EAE (Wilcox et al., 1990; Cannella et al., 1991; Lassmann et al., 1991; O'Neill et al., 1991). An α-ICAM-1-mAb therapy may directly block the migration of leucocytes through the BBB endothelium. All these mechanisms should reduce Gd-DTPA leakage through the BBB. However, the dressing of the endothelial surface with α-ICAM antibodies may directly alter the BBB function, when the expression of ICAM is upregulated in an inflammatory process. This may occur either by complement mediated endothelial damage or by increased scavenging or capping of antibody dressed endothelial surface components. This may finally lead to an increased tracer transport through the BBB.

There is only one study addressing the question of NMR changes in EAE treated with an antibody against adhesion molecules, using actively induced EAE in guinea pigs (Kent et al., 1995). Kent et al. (1995) recently showed that treatment with an antibody against α-4 integrin significantly decreased clinical and MRI findings as compared to control EAE guinea pigs. The principal difference between active and passive AT-EAE is that in AT-EAE the induction phase is not included but in view of the ultimate tissue inflammation and barrier breakdown both models are comparable (Lassmann et al., 1991; Sloan et al., 1991). The significant and reversible decrease of Gd-enhancement and oedema in these treated guinea pigs indicates that in actively induced EAE a stronger inhibition of BBB breakdown occurs (Karlik, personal communication).

Dissociation of clinical and Gd-enhanced MRI findings has also been observed in studies of MS patients. From serial MRI studies in MS patients with Gd-enhancing MS lesions it is known that after corticosteroid bolus therapy of an acute relapse the BBB is initially closed, but after about 10 days will open again — as evidenced by re-enhancing MS lesions — despite clinical improvement (Barkhof et al., 1991; Miller et al., 1992).

We conclude that great caution should be exercised when interpreting Gd-enhanced MRI in EAE and in MS patients after administration of immunomodulatory drugs. The finding of Gd-enhancement indicative of disease activity might be dissociated from the clinical and pathological documentation of a beneficial effect of the drug. If our interpretation of the prolonged and increased Gd-DTPA leakage through the BBB is correct, it may be a warning that interference with adhesion molecules at the BBB in brain inflammation could be a devastating treatment. This is of special interest at a time when a number of ongoing clinical trials investigating immunomodulatory therapy in MS have chosen Gd-enhancement as a primary outcome criterion of MRI disease activity. On purpose we designed the present MRI study similar to protocols used in clinical MS-MRI studies (Barkhof et al., 1991; Miller et al., 1991, 1992), but assessed $T1$-times pre- and post-Gd-DTPA quantitatively rather than estimating $T1$-changes qualitatively. In the future, it may be more appropriate to develop quantitative dynamic MRI studies of the BBB to detect a partial inhibition of BBB breakdown (Larsson et al., 1990; Kermode et al., 1990; Tofts and Kermode, 1991) rather than to assess Gd-DTPA leakage at an arbitrarily single time point as performed in present clinical MS trials.

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