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Beta-amyloid peptide-induced blood-brain barrier disruption facilitates T-cell entry into the rat brain

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Summary

Activated T-lymphocytes can migrate through the blood-brain barrier (BBB) and are able to invade the central nervous system (CNS). In the present study, we investigated whether disruption of the BBB leads to enhanced T-cell migration into the CNS. Amyloid-beta peptide 25-35 (Aβ) or tumor necrosis factor-alpha (TNFα) were administered into the right common carotid artery of adult male Wistar rats. The agents were administered either alone, or were followed by a cell suspension of exogenously activated T-cells. Rats of other groups received activated or non-stimulated T-lymphocytes only. Sagittal brain sections were analyzed with immunohistochemistry of CD3 to reveal the presence of T-lymphocytes within the CNS parenchyma. Administration of activated T-cells alone led to T-cell migration into the brain. Infusion of either substances (Aβ or TNFα) resulted in T-cell invasion of the CNS even when no exogenous T-cells were added. Infusion of either of the agents together with T-lymphocytes generated a more intense T-lymphocyte migration than in the other groups. Electron microscopic analysis and Evans-blue extravasation studies confirmed parallel disruption of the BBB. Our study demonstrates that Aβ and TNFα induce enhanced T-lymphocyte migration towards the brain. This effect may be attributed at least partly to dysfunctioning of the BBB, but other mechanisms are also possible.

Key words: beta-amyloid peptide – blood-brain barrier – central nervous system – T-lymphocyte – tumor necrosis factor-alpha

Introduction

Although the central nervous system (CNS) is considered to be an immunologically privileged site (Barker and Billingham, 1977; Hauser et al., 1983; Hickey et al., 1985), the interpretation of this concept has been challenged recently. Data suggest that even under normal conditions there is a moderate traffic of haematogenous cells through the blood-brain barrier (BBB; Wekerle et al., 1986; Hickey, 1999; Becher et al., 2000). In immune-mediated illnesses of the CNS, cells of the immune system are permitted to enter the brain in large numbers (Paterson and Day, 1982; Hickey et al., 1985; Traugott et al., 1985; Lassmann et al., 1986; Hickey and

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Kimura, 1987). Both in multiple sclerosis (MS) and in experimental allergic/autoimmune encephalitis (EAE), T-lymphocytes have been found in brain parenchyma during the acute phase of the illness (Traugott, 1985; Lassmann et al., 1986), and it was pointed out that leukocyte entry into the CNS is one of the earliest events in the pathogenesis of immune-modulated neurological diseases (Traugott et al., 1985).

Numerous studies investigated the circumstances under which T-lymphocytes can enter the CNS. Intravenous injection of activated T-cells specific for myelin basic protein (MBP) can induce EAE in rats (Ben-Nun A et al., 1981; Wekerle et al., 1986). Furthermore, it has been proven that activated T-cells are able to enter the CNS even when their activity has been induced by an antigen that does not have a CNS origin. At the same time, non-stimulated T-cells may cross the BBB only in virtually non-detectable amounts. Therefore, it was concluded that T-lymphocyte entry into the brain depends primarily on the activation state of the cells (Wekerle et al., 1986; Hickey et al., 1991; Westland et al., 1999). A hypothesis has been formulated to explain how inflammation develops across an intact BBB (Hickey et al., 1991), but inflammation is always accompanied by a dysfunctioning BBB in neuroimmunological illnesses of the CNS. Thus, events at the BBB are of great importance in these processes (Wekerle et al., 1986; Lassmann et al., 1991; Owens et al., 1998).

The aim of the present study was to investigate whether disruption of the BBB leads to enhanced T-cell migration towards the CNS. We studied effects of the pharmacological agents beta-amyloid peptide (Aβ) and tumor necrosis factor-alpha (TNFα) on the entry of T lymphocytes into the CNS in rats. TNFα is a pro-inflammatory cytokine known to be released in response to infections, trauma or in neurological diseases. Recent studies investigating the effects of TNFα on the BBB described distinct changes in the permeability of the BBB following TNFα administration. In rodents, intravenous infusion and pial superfusion of TNFα led to enhanced BBB permeability (Tsao et al., 2001; Mayhan, 2002). At the same time, in vitro experiments provided additional support by showing that the permeability of cerebromicrovascular endothelial cell monolayers was increased for markers of different molecular weight after exposure to TNFα (Mark and Miller, 1999).

Interestingly, TNFα also promoted lymphocyte trafficking throughout the BBB. Both adhesion and transmigration of T-cells were shown to be stimulated by TNFα (Lossinsky et al., 1991; McCarron et al., 1993; Wong et al., 1999). For example, TNFα treatment remarkably upregulated expression of the adhesion molecule ICAM-1 in brain-derived endothelial cells, which coincided with increased T-cell attachment (McCarron et al., 1993). Parallel morphological studies described that T-cells migrated across CNS endothelium by extending finger-like projections into the endothelial cytoplasm and moving either through the endothelial cytoplasm or along intercellular junctions (Lossinsky et al., 1991; Wong et al., 1999). Thus, TNFα was shown to open up the BBB for circulating macromolecules and stimulate transmigration of T-lymphocytes as well.

Similar effects of Aβ on the BBB have been observed. Aβ is a toxic agent accumulating in the form of senile plaques in Alzheimer brains and can also occur in the circulation (Seubert et al., 1982; Hardy and Higgins, 1992; Greenberg et al., 2000; Ertekin-Taner et al., 2001). In order to determine whether Aβ can induce BBB leakage, Aβ fragments such as Aβ(1–40) or Aβ(1–42) were infused either intravenously, or directly into the carotid arteries of rats. As a result, substantial IgG staining and massive Evans blue extravasation was observed in brain parenchyma indicating BBB leakage (Jancsó et al., 1998; Su et al., 1999).

Clear evidence does not yet exist that Aβ can usher the entrance of circulating leukocytes into brain parenchyma. However, compelling results were obtained by employing an *in vitro* BBB model. The experiments showed that Aβ(1–42) induced TNFα secretion by human monocytes, and facilitated monocyte transmigration through the constructed BBB model (Fiala et al., 1998). A follow-up study identified not only monocytes but also T-cells in cerebral perivascular spaces of Alzheimer's disease brains (Fiala et al., 2002). These data indicate that Aβ may be able to induce infiltration of leukocytes and possibly T-cells into the CNS in addition to its capacity to open up the BBB. Since there is evidence that Aβ also induces an inflammatory reaction in the brain and may cause the release of various cytokines including TNFα (Barger et al., 1995; Meda et al., 1995; Forloni et al., 1997; McRae et al., 1997), comparison of the effects of these 2 compounds may provide additional evidence of mechanisms of lymphocyte trafficking into the brain.

Material and methods

Animals and surgical procedures

Adult male Wistar rats (300–350 g) were assigned to 6 experimental groups. Each group contained 5 animals, one animal for each survival time. The animals were anaesthetized with chloralose-urethane solution (50 mg/kg α-chloralose; Sigma, St. Louis MO, USA – 1 g/kg urethane; Hungaropharma, Budapest, Hungary) administered intraperitoneally. Rectal temp was determined continuously with a rectal thermometer and was maintained between 37.0 °C and 37.5 °C with a thermophore.

A midline incision was made on the neck followed by tracheotomy and trachea cannulation. The right common carotid artery was exposed and a fine polyethylene cannula (PE 10) was inserted in the vessel. The pharmacological compounds and/or cell suspensions (see below) were introduced via the cannula in a total volume of 1 ml, at a rate of 0.1 ml/min. Cell suspensions were administered at 10 min after infusion of the pharmacological compounds. The experimental groups and the compounds that were administered are listed in Table 1. After a survival time of 30 min, 1, 2, 3 or 4 h, animals were perfused transcardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA in 0.1 M PBS, pH 7.4). The brains were removed and postfixed in the fixative for 24 h at 4 °C.

Three additional animals were perfused with PBS followed by 2% PFA and 2.5% glutaraldehyde for electron microscopical analysis. Two more animals received Evans blue dye (Sigma) intravenously (50 mg/kg) at 20 min before administration of Aβ or TNFα to show their effects on the permeability of the BBB. Extravasation of Evans blue-albumin-complexes was revealed by fluorescence microscopy (Nikon, Tokyo, Japan).

Pharmacological agents and cell suspensions

Amyloid β-peptide (25–35) (Aβ) was a gift of Prof. B. Penke. The peptide was solubilized in 1–2 drops of 35% acetonitrile and then diluted in PBS. One ml aliquots of 10^{-3} M Aβ were frozen and stored at –20 °C until use. Twenty four h before infusion, the aliquot was thawed and aged for 24 h at 4 °C. Before administration, the aliquot was diluted to a concentration of 5×10^{-4} M and the solution was infused into the right common carotid artery in a volume of 1 ml at a rate of 0.1 ml/min.

Tumor necrosis factor-alpha (TNFα; human recombinant; Sigma) was solubilized in PBS. Animals received an intracarotid infusion of 2.5 µg/kg TNFα in 1 ml total volume at a rate of 0.1 ml/min.

Table 1. Experimental groups and compunds admnistered into the right common carotid artery

Groups	Agents
PHA–	1 ml non-stimulated lymphocyte suspension
PHA+	1 ml PHA-stimulated lymphocyte suspension
Aβ-	1 ml 5×10^{-4} M amyloid β-peptide
TNFα-	2.5 µg/kg TNFα in 1 ml volume
Aβ/PHA+	1 ml 5×10^{-4} M amyloid β-peptide followed by 1 ml PHA-stimulated lymphocyte suspension
TNFα/PHA+	2.5 µg/kg TNFα in 1 ml followed by 1 ml PHA-stimulated lymphocyte suspension

Cell suspensions of allogenic Wistar rat spleen were prepared in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum and incubated in a CO_2 incubator. Cells were stimulated with 10 µg/ml phytohemagglutinin (PHA; Sigma) for 48 h. After incubation, a suspension of $5–10 \times 10^6$ cells/ml was prepared in RPMI 1640 medium containing 1% inactivated rat serum. Cell suspensions were cultured in the same way without PHA-stimulation and served as controls.

To demonstrate the presence of T-lymphocytes in the splenocyte suspension and to assess their approximate number, we employed flow cytometric analysis. Flow cytometry was performed on a FACStar Plus cell sorter (Becton Dickinson, San Diego CA, USA) with FITC-conjugated mouse anti-rat CD3 monoclonal antibodies (Becton Dickinson). The analysis was carried out on 10, 000 cells using the CellQuest 3.1 program (Becton Dickinson). Control measurements, where no antibody was added to the cells did not produce fluorescence. When incubated with FITC-conjugated anti-CD3 antibodies, the cell cultures showed CD3 expression in 20.3% of the cells and 48.4% of the lymphocytes. This means that suspensions of $5–10 \times 10^6$ splenocytes per ml contained approx. $1–2 \times 10^6$ T-lymphocytes per ml. Absence of fluorescence when mouse IgG_1 or mouse serum were added indicated that nonspecific antibody binding did not occur.

Immunohistochemical analysis

Postfixed brains were embedded in paraffin and 4-µm-thick serial sagittal sections were cut. After deparaffinization and endogenous peroxidase blockade we used target retrieval solution (Dako, Glostrup, Denmark) for antigen retrieval. Nonspecific binding sites were blocked with skim milk powder. Sections were then incubated in the presence of the primary antibody (rabbit anti-human; Dako). The secondary antibody was horseradish peroxidase-conjugated anti-rabbit (EnVision™; Dako). Peroxidase activity was visualized using DAB. Cell nuclei were counterstained with haematoxylin.

Sections from the brain of one animal were analyzed semiquantitatively in each group. Ten sections from each brain were examined and T-cells in brain parenchyma were counted using a light microscope (E600; Nikon). Brain regions examined included cortex, diencephalon, hippocampus, mesencephalon, olfactory tract and pineal gland. Numbers of T-lymphocytes were evaluated using repeated measurement ANOVA.

Electron microscopical analysis

Additionally, 3 rat brains were prepared for electron microscopical analysis. The animals received a unilateral intracarotic infusion of either PBS, TNFα or Aβ according to the protocol above without infusing exter-

nal lymphocytes. After a 2 h-survival time, rats were transcardially perfused with PBS followed by a solution of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M PBS (pH 7.4). Brains were removed and stored overnight in fixative at 4 °C.

The hippocampus and frontoparietal cortex of the ipsilateral hemisphere, and the pineal gland were prepared for embedding. Samples were dehydrated and embedded in Durcupan epoxy resin (Fluka, Buchs, Switzerland). Semi-thin sections were cut on an ultramicrotome (Ultracut E; Reichert-Jung) and stained on microscopic object glasses with a 1:1 mixture of methylene blue and Azure II blue. Samples were subsequently coverslipped with DPX and analyzed under a light microscope (E600; Nikon). Ultrathin sections were cut and collected on 200-mesh copper grids. Preparations were then contrasted with 5% uranyl-acetate and Reynolds lead-citrate solution. Finally, samples were analyzed using a TM10 transmission electron microscope (Philips, Eindhoven, The Netherlands) and the ultrastructural features of the BBB were characterized.

Results

Immunohistochemistry

Fluorescence microscopy showed extravasation of Evans-blue-albumin-complexes into the nervous tissue after administration of TNFα or Aβ (Fig. 1). Immunofluorescence of the Evans-blue dye verified disruption of the BBB after administration of both TNFα and Aβ. Infiltration of the dye into the CNS was not detected in the control group, which indicates the presence of an intact BBB.

Sagittal brain sections labelled with anti-CD3-antibodies were analyzed for T-cell invasion. In sections showing CD3 positivity, most T-lymphocytes were found in the subpial cortical grey matter, in perivascular spaces, in specific groups deeper in the brain parenchyma and in periventricular regions. The subarachnoid space and the pineal gland – regions without BBB – contained numerous T-cells in each group – except when non-stimulated T-lymphocytes were administered

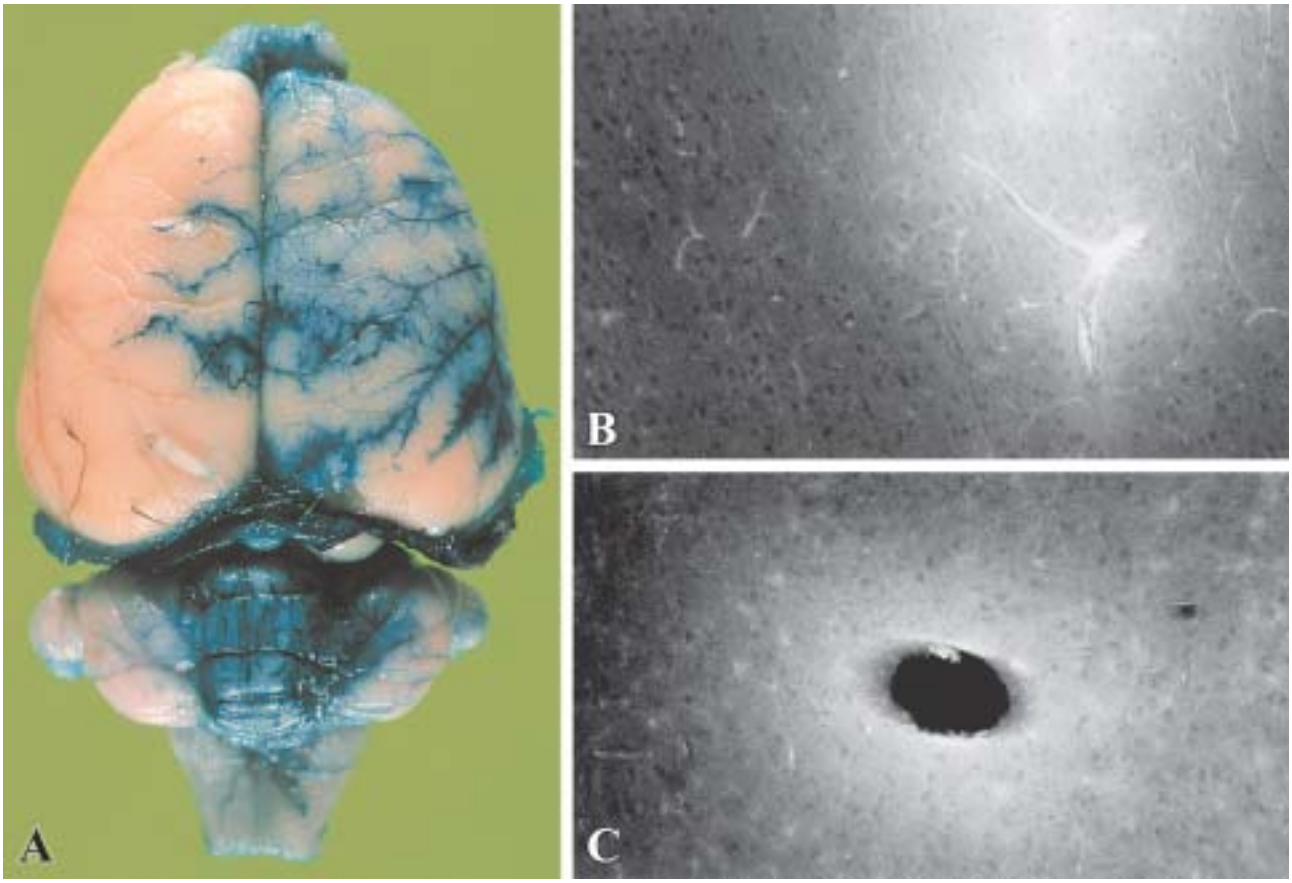


Fig. 1. Evans-blue extravasation in the brain at 30 min after $A\beta_{25-35}$-infusion into the right common carotid artery. **A**. Macroscopic appearance of Evans-blue extravasation. **B, C**. Fluorescence of Evans-blue dye around a cerebral vessel after extravasation. Magnifications, B, ×20; C, ×40.

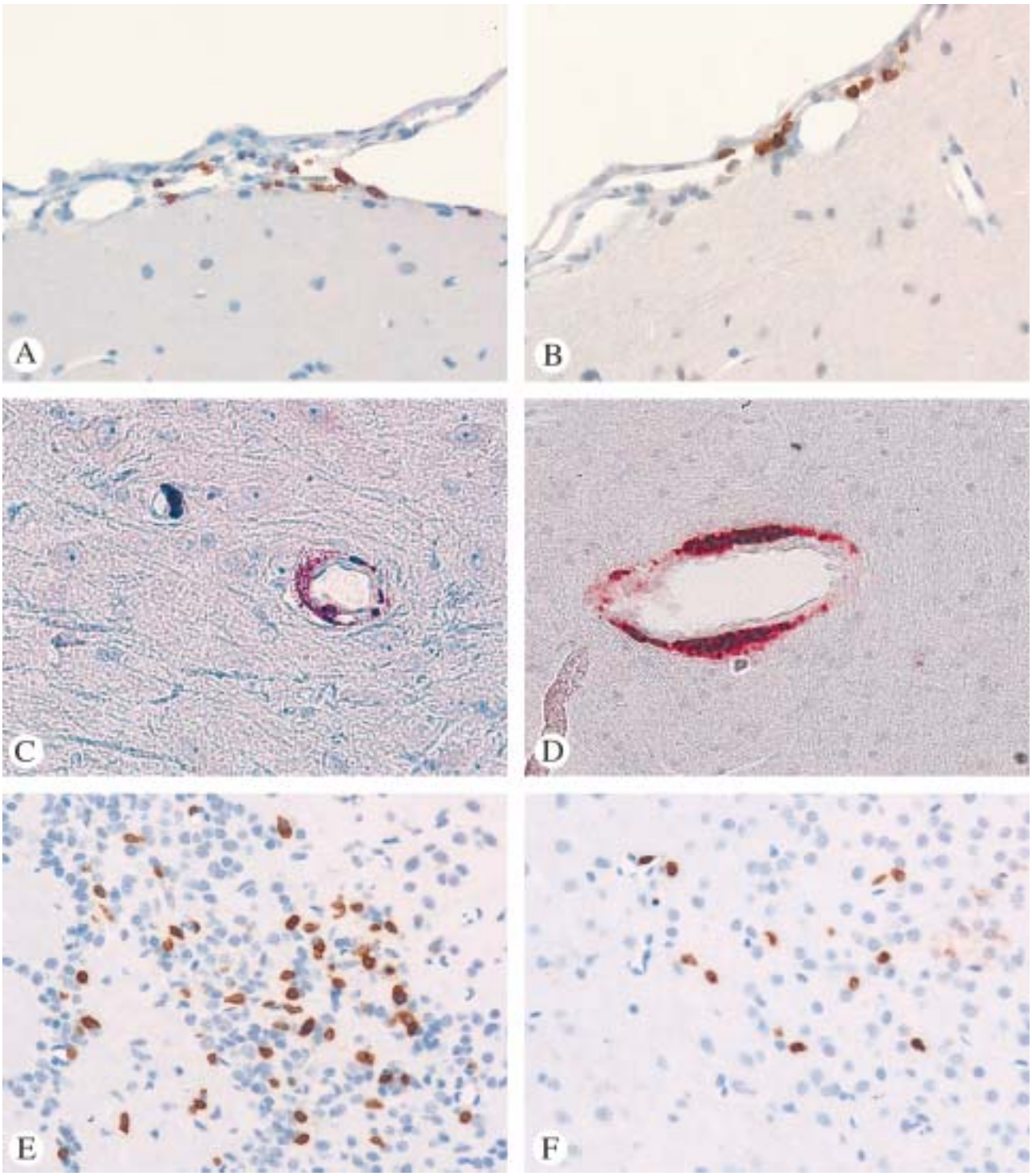


Fig. 2. Photomicrographs of CD3-positive T-lymphocytes in rat brain. **A**. T-cells in the subpial cortex at 30 min after TNFα treatment. **B**. Subpial T-lymphocytes at 60 min after Aβ-infusion. **C**. Perivascularly localized T-lymphocytes in the cortex at 60 min after infusion of TNFα and an exogenous T-cell suspension. **D**. T-lymphocytes around a cortical blood vessel at 4 h after treatment with Aβ and administration of a T-cell suspension. **E**. T-cells in the olfactory tract at 3 h after infusion of TNFα. **F**. T-lymphocytes in the diencephalon at 4 h after infusion of Aβ. Magnification, ×40.

alone. Regional distribution patterns of T-cells were similar in all groups, when T-cells were present in the CNS.

After administration of nonstimulated T-lymphocytes (group PHA-), lymphocytes were not found in CNS parenchyma.

As expected, activated T-lymphocytes penetrated into the CNS. Sections from group PHA+ showed CD3-positive cells scattered in the brain parenchyma. Comparison of the results of groups PHA+ and PHA- indicated that activated T-lymphocytes entered the CNS, whereas non-stimulated T-cells were unable to infiltrate brain parenchyma (Fig. 3).

T-cells were also found in various regions of the CNS when Aβ was administered alone (group Aβ-; Fig. 2B, 2F). Similarly, T-cells were detected intracerebrally after TNFα injection when an exogenous cell suspension was not administered (group TNFα-; Fig. 2A, 2E). Interestingly, both Aβ treatment and TNFα treatment resulted in T-cell invasion into the CNS even when an exogenous T-cell suspension was not administered. T-cells were scattered in similar brain regions in both cases. The numbers of cells found in the CNS were moderately higher in the Aβ-group, especially at the 2nd and 3rd h postinjection (Fig. 3).

CD3-positive cells were also detected in the CNS when administration of Aβ was followed by an infusion of exogenous PHA-activated T-lymphocytes (group Aβ/PHA+; Fig. 2D). The T-cell distribution pattern was similar to that in case of TNFα infusion. Administration of TNFα and exogenous activated T-lymphocytes (group TNFα/PHA+) resulted in T-cell invasion in perivascular spaces (Fig. 2C) and in brain parenchyma.

When Aβ and activated exogenous T-cells were infused together, T-cells were already found at 30 min after infusion of the cell suspension. Afterwards, numbers of T-lymphocytes continued to increase and were highest at 4 h after infusion. In the 3rd and 4th h postinjection, numbers of T-cells in brain parenchyma exceeded numbers of T-cells when either Aβ alone or activated T-cells alone were administered (Fig. 3B).

Fig. 3A shows that the number of T-cells found in the CNS was highest during the first h postinjection when TNFα and activated T-lymphocytes were infused together. Subsequently, the number of T-cells levelled off and was similar to that found in the TNFα- and the PHA+ group. Thus, in the first h, administration of TNFα and exogenous T-lymphocytes resulted in a stronger T-cell invasion into CNS parenchyma, than after either TNFα treatment alone or administration of activated T-lymphocytes alone. The brain regions where most of the T-cells were found were similar to those in the other groups.

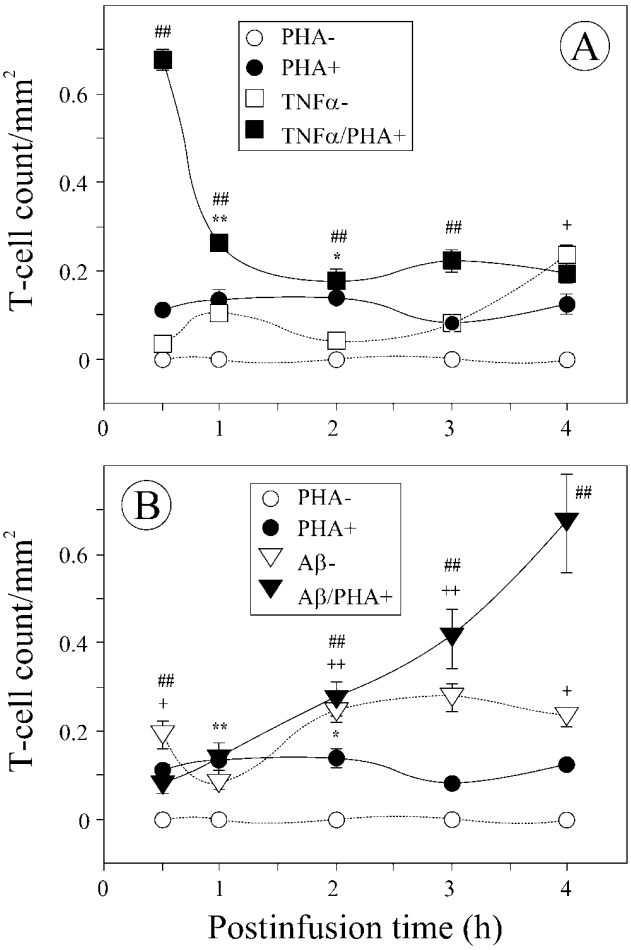
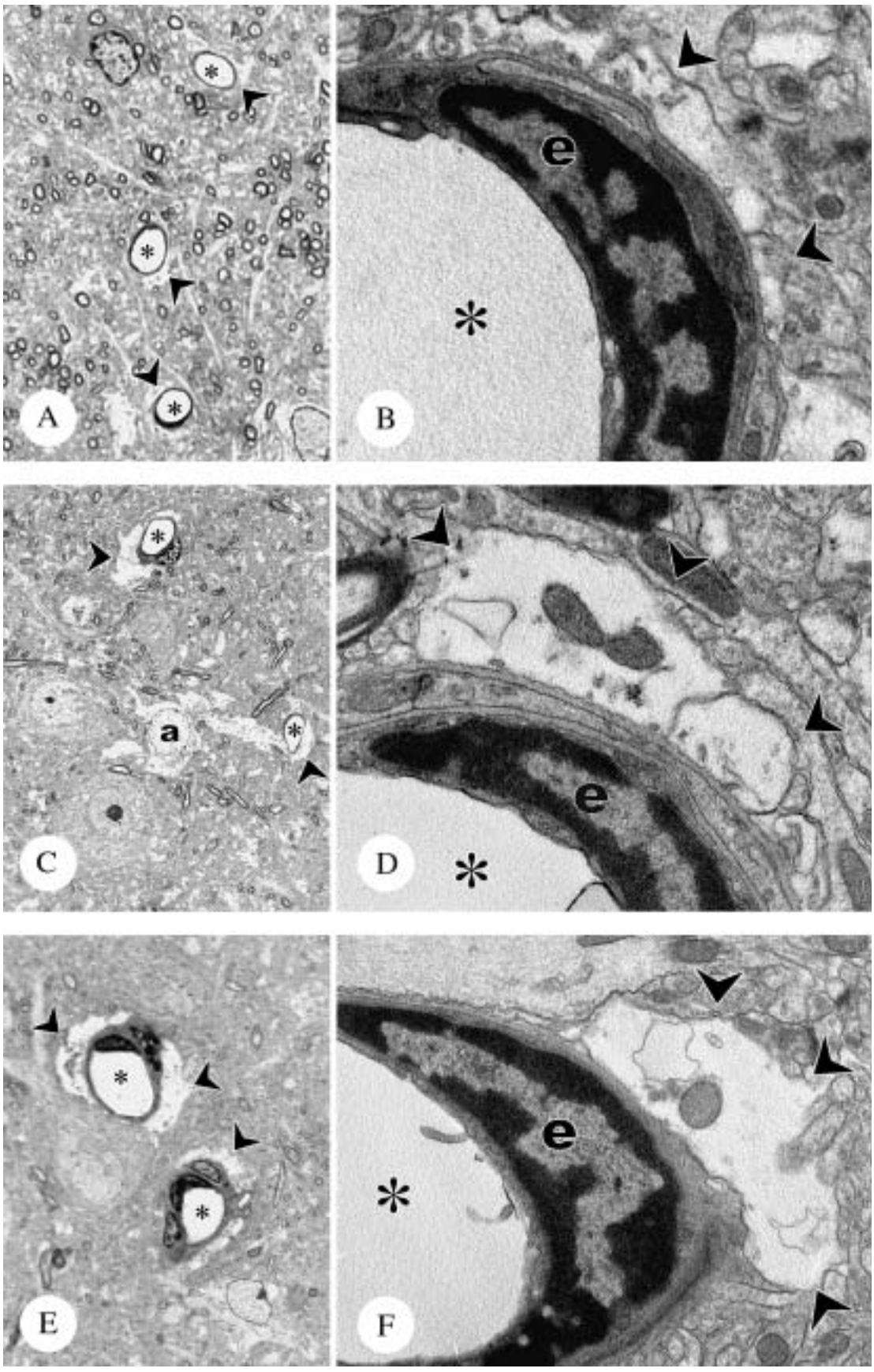


Fig. 3. Time-related alterations in numbers of T-cells per unit area in rat brain. Each symbol depicts the T-cell concentration in the brain as mean (± SEM) of 10 sagittal sections of 1 rat. Repeated measurement ANOVA indicated significant time-dependent and group-related changes ($p < 0.001^{\pm\pm}$). **A**. TNFα treatment. **B**. Aβ treatment. *, PHA- × PHA+; +, PHA- × TNFα-/Aβ-; #, PHA- × TNFα+/Aβ+.

Electron microscopy

Samples of the ipsilateral hippocampus, frontoparietal cortex and pineal gland of one representative animal per group were analyzed by electron microscopy. Since the infused compounds affect permeability and/or integrity

Fig. 4. Swelling of perivascular astrocytic end feet in rat hippocampus after unilateral intracarotic infusion of saline (**A, B**), TNFα (**C, D**) and beta-amyloid$_{25–35}$ (**E, F**), as shown in semi-thin (**A, C, E**) and ultrathin (**B, D, F**) sections. Asterisk, microvascular lumen; a, astrocytic perikaryon; e, microvascular endothelial cell. Arrowheads indicate perivascular astrocytic end feet. Magnifications, A, C, E, ×100; B, D, F, ×7200.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of the BBB, we focused on the ultrastructure of cerebral arterioles and capillaries.

The most obvious morphological change observed in semi-thin sections was the presence of extensive lacunae surrounding microvessels in both hippocampus and frontoparietal cortex after TNFα and $A\beta_{25–35}$ infusion (Fig. 4C, E). The lacunae were identified by electron microscopy as swelling of perivascular astrocytic end feet (Fig. 4D, F). Astrocytic end feet adjacent to the vascular basement membrane covered an increased surface, contained swollen mitochondria in an empty-looking cytoplasm characterized by low electron density after treatment with TNFα and Aβ. Not only end feet but also perikaryon and proximal processes of astrocytes had a swollen appearance.

An irregular luminal endothelial surface characterized TNFα- and $A\beta_{25–35}$-treated animals. Moreover, endothelial cells were swollen, contained a higher amount of pinocytic vesicles and showed occasionally loosened tight junctions particularly after $A\beta_{25–35}$ infusion.

Discussion

The present study has characterized T-cell entry into the CNS in relation to the activated state of lymphocytes and disruption of the BBB. Administration of exogenously activated T-cells alone without disruption of the BBB resulted in T-cell infiltration into brain parenchyma. Since the antigen used for activation of T-lymphocytes was not of CNS origin, such as the frequently used myelin basic protein, the activated state of the lymphocytes rather than antigen specificity appeared to be important for CNS entry. In this respect, our data are in line with previous reports suggesting that activated T-cells can penetrate the CNS irrespective of their antigen specificity or T-cell phenotype (Hickey et al., 1991).

The main goal of the present study was to show whether disruption of the BBB facilitates entry of activated T-cells into brain parenchyma. We have used 2 agents, TNFα and $A\beta_{25–35}$, to disrupt the BBB. Interestingly, both compounds produced moderate T-cell infiltration into the brain when exogenous lymphocytes were not added. Since non-activated T-cells cannot penetrate the BBB (Merrill and Benveniste, 1996), either infused TNFα and $A\beta_{25–35}$ or disruption of the BBB itself prime naive T-cells and facilitate their migration through the BBB. In line with this finding, TNFα in combination with interleukins has been shown to recruit naive T-lymphocytes into the liver (Abrignani, 1998), whereas the presence of Aβ as an infectious agent in the circulation can also stimulate T-cells (Trieb, 1996). However, the damage imposed on the BBB may also contribute to T-cell stimulation for reasons described below.

T-cell counts after TNFα infusion were comparable with T-cell counts after administration of activated T-cells alone, whereas $A\beta_{25–35}$ produced a more distinct increase in T-cell numbers. Therefore, we assume that particularly Aβ infusion imposed such an acute damage to the BBB as was also demonstrated by our electron microscopical study, that it set off a neuroimmune reaction involving T-cells. This suggestion is supported by studies investigating immune responses following cerebrovascular events. For example, experimental cerebral ischaemia or stroke which are accompanied by BBB disruption caused accumulation of T-cells in the affected brain region (Schroeter et al., 1994; Jander et al., 1995; Bona et al., 1999; Campanella et al., 2002). However, these experiments identified neuronal injury rather than BBB damage as initiator of T-cell infiltration. Along this line, neuronal damage itself with an intact BBB also caused T-cell aggregation at the injured CNS site (Raivich et al., 1998). A possible mechanism of Aβ-induced T-cell entry through an intact BBB may involve induction of adhesion molecules such as ICAM and VCAM, which facilitate peripheral monocyte transmigration through cultured brain endothelial cell monolayers (Giri et al., 2000, 2002). Therefoe, the cerebrovascular event induced in our study may have had similar consequences with respect to neuroimmune reactions as focal cerebral ischaemia or stroke.

Our data demonstrate that a combination of TNFα or $A\beta_{25–35}$ with primed exogenous T-cells most effectively accelerated T-cell entry into forebrain structures. These findings support our original hypothesis that BBB disruption can promote activated T-cell infiltration into the CNS. However, the temporal pattern of T-cell infiltration appeared to be inverse when comparing the effects of TNFα and $A\beta_{25–35}$. TNFα caused strongest T-cell accumulation at 30 min after infusion, whereas $A\beta_{25–35}$ showed a delayed action with a peak at 4 h. These complementary data confirm the suggestion of Rhodin and Thomas (2001) that the inflammatory process set off by circulating Aβ is mediated by TNFα. Experiments leading to this conclusion showed prevention of Aβ-induced leukocyte invasion into peripheral tissue by administration of TNFα-binding protein (Sutton et al., 1999; Rhodin and Thomas, 2001). Moreover, the temporal pattern of Aβ-induced leukocyte migration appeared to be similar to that found in our study.

Since Aβ is known to be a prominent neurotoxic peptide playing a central role in Alzheimer's disease, our data may have implications in the aetiology of such neurodegenerative disorders. A recent study demonstrated that besides monocyte/granulocyte infiltration, T-lymphocytes frequently occurred in Alzheimer brains (Fiala et al., 2002). Because Alzheimer's disease is linked with an array of neuroinflammatory signals (Akiyama et al., 2000; Cooper et al., 2000), the results presented here may contribute to the understanding of cerebral inflammatory processes induced by Aβ.

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