

Production of neuroprotective NGF in astrocyte–T helper cell cocultures is upregulated following antigen recognition

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Received 13 August 2003; received in revised form 7 November 2003; accepted 15 December 2003

Abstract

Astrocytic production of nerve growth factor (NGF) is increased during inflammation of the central nervous system (CNS). Here we show that cell–cell interaction between primary murine astrocytes and myelin basic protein (MBP)-specific T cell receptor (TCR) transgenic Th1 and Th2 cells significantly increased production of NGF. This upregulation was found to be dependent on antigen recognition. Neutralization of cytokines produced in cocultures did not affect NGF production. This novel finding suggests a neuroprotective role of astrocytes during T cell-mediated inflammation in the CNS.

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Keywords: Th1; Th2; Growth factors; Neurotrophins; Mouse

1. Introduction

Nerve growth factor (NGF) constitutes one member of the neurotrophin family. Growth, differentiation, survival, and maintenance of peripheral and central neurones are functions facilitated by NGF (Levi-Montalcini et al., 1996). It is known that administering NGF intracerebroventricularly into marmosets delays onset of experimental autoimmune encephalomyelitis (EAE) and reduces lesion formation (Villoslada et al., 2000). Subsequent to induction of EAE, mice treated with NGF by intraperitoneal injection exhibited a delayed onset of disease in combination with lower clinical scores under the duration of the disease (Arredondo et al., 2001). Moreover, myelin basic protein (MBP)-specific T cells retrovirally transduced to secrete high levels of NGF are unable to mediate clinical EAE and suppress induction of EAE by nontransduced MBP-specific T cells in rats (Flügel et al., 2001). Evidently, NGF can act as a protective agent in neuro-inflammatory diseases in animal models.

The major source of NGF in the central nervous system (CNS) is represented by astrocytes (Eddleston and Mucke, 1993). Other glial cells in the CNS such as microglia (Elkabes et al., 1996) and oligodendrocytes (Du and Dreyfus, 2002) are also capable of producing NGF. Furthermore, Th2 cells (Ehrhard et al., 1993; Arredondo et al., 2001) and, to a lesser extent, Th1 cells (Santambrogio et al., 1994) themselves are, to a certain extent, capable of producing NGF. Cells from both the nervous system and the immune system express the functional NGF receptor TrkA, suggesting that NGF participates in the interactions between these two systems (Levi-Montalcini et al., 1996).

Astrocytes are important for the physiological homeostasis in the CNS, in that they can affect and regulate neuronal function (Dong and Benveniste, 2001). During inflammation in the CNS, they could be involved in interacting with invading T cells. Astrocytic major histocompatibility complex (MHC) class II expression is seen during prolonged inflammation in vivo (Kreutzberg, 1996) and after stimulation, for example, by interferon- γ (IFN- γ) in vitro (Aloisi et al., 1998). Consequently, antigen presentation to CD4⁺ T cells is possible (Fontana et al., 1984). The astrocytes are, however, not as efficient as splenic antigen-presenting cells (APCs) to activate Th1 and Th2

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cells. Moreover, they are more effective in restimulating Th2 cells than Th1 cells (Aloisi et al., 1998). Th1 cells typically produce the pro-inflammatory cytokine IFN- γ (Mosmann and Sad, 1996), while Th2 cells exhibit an anti-inflammatory cytokine profile, such as interleukin (IL)-4 and IL-10. These two cytokines have, in contrast to IFN- γ , been shown to be potent inducers of NGF in astrocytes (Brodie, 1996; Brodie et al., 1998). To investigate how astrocytes respond to an inflammation within the CNS in terms of NGF production, we used a coculture system with astrocytes and MBP-specific Th1 or Th2 cells. In this study, we have demonstrated an increased production of NGF in cocultures of murine astrocytes and Th1 or Th2 cells, which was mediated by TCR–MHC class II interaction, suggesting a neuroprotective role of astrocyte–T cell interactions.

2. Materials and methods

2.1. Materials

Recombinant human IL-2 was purchased from Chiron (Ratingen, Germany), recombinant mouse IL-4 was purchased from Biosource International (CA, USA), recombinant mouse IFN- γ was obtained from PeproTech (Frankfurt am Main, Germany), and recombinant mouse IL-12 was obtained from R&D Systems (Wiesbaden, Germany). Anti-mouse ICAM-1 (clone KAT-1) and anti-mouse VCAM-1 (clone 6C71) antibodies, neutralizing anti-mouse IFN- γ (clone AN18), and anti-mouse IL-10 (clone JES5-2A5) antibodies were generously provided by the Deutsches Rheuma-Forschungszentrum (Berlin, Germany). Neutralizing anti-mouse IL-4, anti-mouse IL-6, and anti-mouse IL-12 antibodies were acquired from BD Pharmingen (Heidelberg, Germany), and neutralizing anti-mouse IFN- α/β was acquired from Biosource International. MBP peptide Ac1-11 was purchased from Affina Immunotechnik (Berlin, Germany). OVA peptide 323–339 was obtained from TIB/MolBiol (Berlin, Germany).

2.2. Animals

Transgenic mice, carrying a T cell receptor (TCR) for MBP peptide Ac1-11 on a B10.PL background, were kindly provided by David Wraith (University of Bristol) (Liu et al., 1995). Wild-type B10.PL mice were purchased from Jackson Laboratories (Bar Harbor, ME). The animals were kept under standard laboratory conditions of 24 °C and 12–12 h light–dark cycle in accordance with regulations for proper animal care. Transgenic DO10 mice, carrying a TCR for ovalbumin (OVA) peptide 323–339 on a BALB/c background, were kindly provided by Monika Brunner-Weinzierl (Berlin). Wild-type BALB/c mice were obtained from Harlan Winkelmann (Borchen, Germany). Adult mice were sacrificed under ether anesthesia by cervical dislocation, and neonatal mice by decapitation.

2.3. Astrocyte cultures

Primary cortical astrocytes were prepared from 1-day-old B10.PL mice as described by Hertz et al. (1989). Briefly, frontal cortices were gently taken out from the brain, and subsequently mechanically dissociated through a mesh (80 μ m) prior to the cells being seeded into poly-L-lysine-coated (Sigma, Taufkirchen, Germany) culture dishes (six-well plates). Cells were incubated in DMEM (Gibco BRL, Paisley, UK) supplemented with 10% foetal bovine serum (Biochrom, Berlin, Germany), 100 U/ml penicillin/streptomycin (Sigma), and 2.5 mM L-glutamine (Gibco BRL). The medium was exchanged after 1 day and then every 3–4 days. T cells were added when astrocytes had reached a confluent monolayer 12–14 days later. The culture consisted of >95% pure astrocytes as determined by glial fibrillary acidic protein fluorescein isothiocyanate (GFAP-FITC; Sigma) and CD11b-PE (BD Pharmingen) staining of contaminating microglia (Fig. 1A). In order to activate the astrocytes, they were incubated with 100 U/ml IFN- γ for 24 h prior to the experiment. At the day of adding T cells to the astrocyte cultures, the medium was exchanged, and in addition, the IFN- γ -pretreated astrocytes were washed to

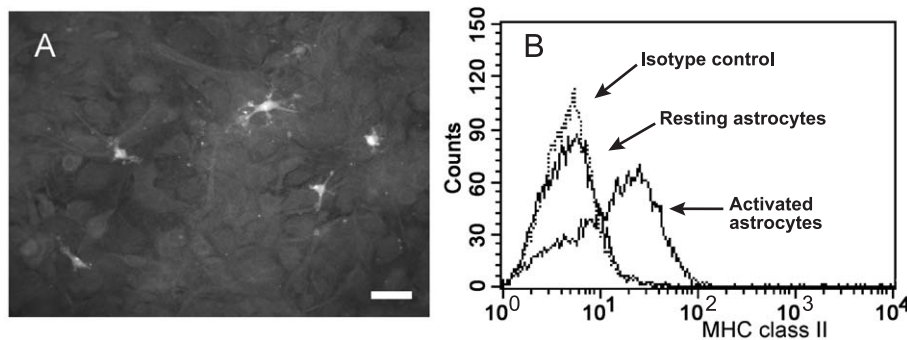


Fig. 1. Characterization of astrocyte cultures. (A) CD11b-PE staining of microglial contamination in astrocyte cultures. The purity of astrocytes was >95%. Scale bar=40 μ m. (B) MHC class II expression on activated astrocytes, resting astrocytes, and control isotype (dotted line).

remove IFN- γ completely. For fluorescence-activated cell scanning (FACS) analysis, the astrocytes were detached by trypsination and stained with anti-MHC class II (clone Y-3P, FITC-conjugated; ATCC) and CD11b-PE antibodies. Respective isotype controls were rat IgG2a-FITC and rat IgG2b-PE. The gate was set on living CD11b-negative cells.

2.4. T cell lines

For establishing MBP-specific Th1 and Th2 cell lines, a mixture of spleen and lymph node cells (2×10^6 cells/ml) of adult MBP transgenic mice was cultured in RPMI 1640 (Gibco BRL) supplemented with 10% foetal bovine serum (Biochrom), 100 U/ml penicillin/streptomycin (Sigma), 2 mM L-glutamine (Gibco BRL), and 50 μ M 2-mercaptoethanol (Merck, Darmstadt, Germany) with 3 μ g/ml MBP peptide in the presence of either recombinant mouse IL-12 (1 ng/ml) plus anti-mouse IL-4 (1 μ g/ml), or recombinant mouse IL-4 (200 U/ml) plus anti-mouse IL-12 (1 μ g/ml) to prime for Th1 or Th2 cell lines, respectively. After 3 days, T cells were expanded in culture medium with the addition of recombinant human IL-2 (100 U/ml). After 7 days, T cells (10^6 cells/ml) were restimulated with irradiated splenocytes (10^6 cells/ml) of B10.PL mice as APCs plus 3 μ g/ml MBP peptide and the respective cytokines and antibodies for Th1 and Th2 cells. Supernatants of the cell lines were tested for their Th1 and Th2 cytokine content by OptEIA™ enzyme-linked immunosorbent assay (ELISA) kits for IFN- γ and IL-4 (BD Pharmingen), respectively, at 24 and 48 h after restimulation. OVA-specific Th cells were prepared in the same manner, except for the omission of Th1- or Th2-polarizing conditions. OVA peptide was added to cultures at a concentration of 1 μ g/ml. These T cells were restimulated using splenic APCs from BALB/c mice. Twenty-four hours after restimulation, CD4⁺ T cells were isolated by magnetic cell sorting (anti-CD4 MACS MicroBeads; Miltenyi Biotec, Bergisch-Gladbach, Germany). FACS analysis showed approximately 95% CD4⁺ cells. T cells were washed in DMEM before they were added to the astrocytes in a concentration of approximately $3.3\text{--}10^5$ cells/ml. The T cell–astrocyte ratio was about 1:4.

2.5. Cocultures

In cocultures with cell–cell contact between T cells and astrocytes, the T cells were gently added directly onto the confluent monolayer of astrocytes. In cocultures where no contact between T cells and astrocytes was required, a cell culture insert with a pore size of 0.4 μ m (Falcon, BD Franklin Lakes, NJ, USA) was placed in the well above the astrocytes, and T cells were added to the medium above the membrane. T cells and astrocytes then shared the medium. The concentration of MBP peptide was 3 μ g/ml in all experiments. Cell-free supernatants were collected after 24 h and kept frozen until subsequent analysis by ELISA.

2.6. Cytokines, growth factor detection, and cross-linking of adhesion molecules

NGF levels in supernatants from T cells, astrocytes, and cocultures were detected by ELISA (Promega, Mannheim, Germany). For detection of cytokines such as IL-4, IL-6, IL-10, tumor necrosis factor (TNF)- α , and IFN- γ , OptEIA™ ELISA kits from BD Pharmingen were used. IL-1 β and transforming growth factor (TGF)- β ELISA kits were obtained from R&D Systems. When using neutralizing antibodies, they were added to the cocultures at a concentration of 10 μ g/ml. Cross-linking the adhesion molecules ICAM-1 and VCAM-1 was performed by incubating activated astrocytes with the respective rat anti-mouse antibodies (20 μ g/ml) for 30 min at room temperature. Subsequent to washing, the antibodies were cross-linked by addition of anti-rat IgG (Dianova, Hamburg, Germany). The astrocytes were kept for 24 h in culture before the supernatants were collected.

3. Results

NGF production was investigated in a setting that mimics the CNS environment during a T cell-mediated inflammation. To this end, primary astrocyte cultures were prepared from neonatal mice. Fig. 1A shows the purity of these cultures, demonstrating a very low microglial contamination. In order to allow for antigen presentation by astrocytes, they were activated by IFN- γ . Flow cytometry confirmed IFN- γ -induced MHC class II expression in our cultures (Fig. 1B). To determine NGF production by astrocytes in the absence of T cells and to find out whether T cells themselves could be a source of NGF, astrocytes and T cells were cultured separately. In addition, T cells were cocultured with APCs and antigen. Fig. 2A shows that Th1 (40 ± 11 pg/ml, mean \pm S.E.M.) and Th2 (50 ± 10 pg/ml) cells produced significantly less NGF ($p < 0.005$) than astrocytes (411 ± 64 pg/ml). The same could be observed for Th1 (45 ± 18 pg/ml) and Th2 (48 ± 13 pg/ml) cells, together with splenic APCs and MBP peptide.

When Th1 or Th2 cells were cocultured with activated astrocytes in the presence of MBP peptide, and direct contact between the astrocytes and T cells was possible, a significant upregulation ($p < 0.05$) of NGF was detected in both of the cocultures by factors of 2.3 ± 0.3 and 2.0 ± 0.2 , respectively (mean \pm S.E.M.), compared to astrocytes cultured alone (control, Fig. 2B). In the absence of MBP peptide, no upregulation of NGF was observed. Moreover, in the cocultures of resting astrocytes that did not express MHC class II (see Fig. 1B), and thus did not present antigen to the T cells, no significant upregulation of NGF was detected compared to the control (Fig. 2C). This observation was independent of the presence of MBP peptide (data not shown) and in agreement with what intuitively would be expected. Astrocytes produced the same levels of NGF both

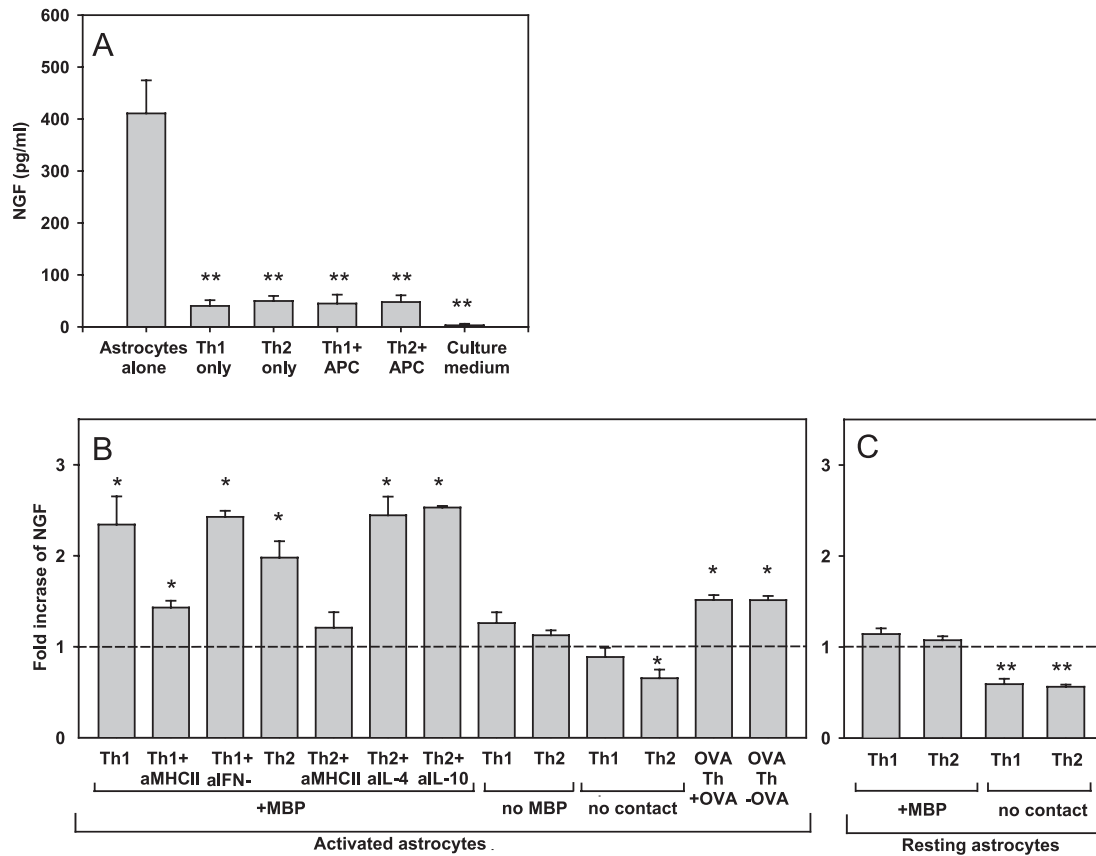


Fig. 2. Levels of NGF produced by astrocytes. (A) NGF production by Th1 or Th2 cells cultured alone or with splenic APCs and MBP peptide in the absence of astrocytes, and astrocytes cultured alone. (B) Astrocytes were incubated with IFN- γ (100 U/ml) for 24 h to upregulate MHC class II. The levels of NGF in the cocultures were compared to the NGF levels produced by astrocytes alone (control). In the figure, the control level is set to unity, indicated by the dashed horizontal line. The Th1 or Th2 cells were cocultured with astrocytes either with cell–cell contact, or with a membrane separating them from the astrocytes. Neutralizing IFN- γ , IL-4, or IL-10 in the cocultures did not reduce NGF production. Blocking MHC class II, however, resulted in reduced NGF production. A similarly low NGF production was observed when coculturing astrocytes with OVA-specific T helper cells (OVA T) from a different MHC class II background in the presence or absence of antigen (OVA). Here TCR–MHC class II interaction was impossible. (C) Resting astrocytes (not treated with IFN- γ) cocultured with Th1 or Th2 cells.

Data are expressed as mean n -fold increase compared to control \pm S.D. from two independent experiments with neutralizing anti-cytokine antibodies and OVA-specific T cells, respectively. The remaining data are expressed as mean n -fold increase compared to control \pm S.E.M. from five independent experiments. * p < 0.05 and ** p < 0.005 by paired Student's t test.

in the presence and absence of MBP peptide (data not shown). When adding naïve CD4⁺ T cells to the astrocytes, no change in the level of NGF was detected (data not shown).

In order to investigate the effect of soluble factors as opposed to cell–cell interactions, direct contact between the T cells and the astrocytes was prevented. This was achieved by inserting a membrane into the culture dish to physically separate the two different cell types. The T cells and the astrocytes could then communicate only via soluble factors. When Th1 cells were added to the medium above the membrane in the cultures of activated astrocytes, the NGF levels were slightly, but not significantly, lower than control levels. Contrary to this, and rather surprisingly, the NGF level was found to be significantly lower (p < 0.05) when Th2 cells were added to this system (factor 0.65 ± 0.10 ; Fig. 2B). Furthermore, NGF levels were significantly lower (p < 0.005) in the presence of a membrane between the

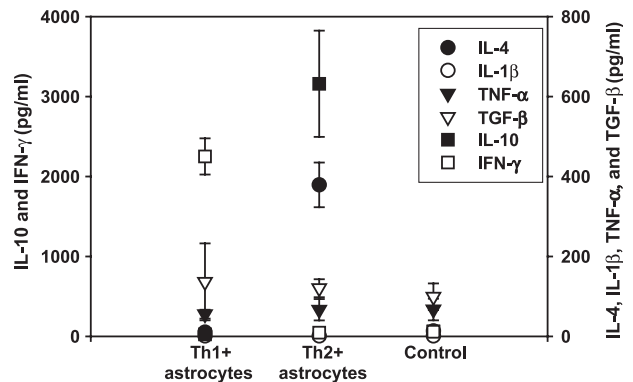


Fig. 3. Cytokines in cocultures of activated astrocytes and T cells. Contact between T cells and astrocytes was allowed in all of these cocultures. The control was activated astrocytes cultured in the absence of T cells. MBP peptide was present in all cultures. Data are expressed as mean \pm S.E.M. from three individual experiments.

resting astrocytes and Th1 and Th2 cells than in the contact situation and the control (0.59 ± 0.06 and 0.56 ± 0.02 , respectively; Fig. 2C). To verify that this lower level of NGF in the cocultures was not a trivial artefact of the mere presence of the membrane, such membranes alone were inserted to astrocyte cultures. This did not affect NGF levels (data not shown). Moreover, NGF levels from T cells incubated in astrocyte-conditioned medium (ACM) were the same as in ACM incubated alone, but lower than NGF levels in the ACM prior to this incubation. From this, NGF consumption by T cells was excluded.

It is well known that there exist potent inducers of NGF production by astrocytes, such as IL-1 β , TNF- α (Brodie, 1996), and TGF- β (Hahn et al., 1997). The cocultures were therefore screened for these cytokines and growth factors (Fig. 3), but no correlation was found to the observed NGF level. Other known strong inducers of NGF in astrocytes such as IL-4 (Brodie et al., 1998) and IL-10 (Brodie, 1996) were found to be upregulated in the cocultures of Th2 cells and activated astrocytes when MBP peptide was present (379 ± 3 and 3160 ± 664 pg/ml, respectively; Fig. 3). However, neutralization of IL-4 and IL-10 yielded no reduction of NGF production (Fig. 2B)—neither did neutralization of IL-6 nor IFN- β (data not shown), which are also known as potent inducers of NGF production in astrocytes (Kossmann et al., 1996; Boutros et al., 1997). IFN- γ has not only been shown not to upregulate NGF production in astrocytes but even to suppress IL-10-induced NGF production (Brodie, 1996). Nevertheless, since IFN- γ was the major product in cocultures of astrocytes and Th1 cells (2251 ± 226 pg/ml; Fig. 3), IFN- γ was neutralized and, as expected, the level of NGF was not reduced (Fig. 2B). These results suggest that the upregulation of NGF in the cocultures is not due to the cytokines produced following T cell activation, but rather to the TCR–MHC class II interaction itself. Additionally, when blocking MHC class II on astrocytes with a monoclonal antibody, astrocytes did not upregulate NGF to the same extent as without blocking (Fig. 2B). Support for the responsibility of TCR–MHC class II interaction came from MHC class II mismatch experiments (i.e. cocultures of B10.PL astrocytes with OVA-specific Th cells on a BALB/c background). Under these conditions, TCR–MHC class II interactions could be excluded. Indeed, we found an NGF production similar to that seen with MHC blocking. NGF production was only slightly increased in these cultures independent of the presence of OVA peptide (Fig. 2B). In summary, these findings suggests that TCR–MHC class II interactions per se play a crucial role in upregulation of NGF levels and this upregulation is independent of the T helper cell phenotype.

Alternatively, the upregulation of NGF could be due to cellular interactions subsequent to TCR–MHC class II interaction. To further address this point, we studied the role of adhesion molecules that are expressed on astrocytes.

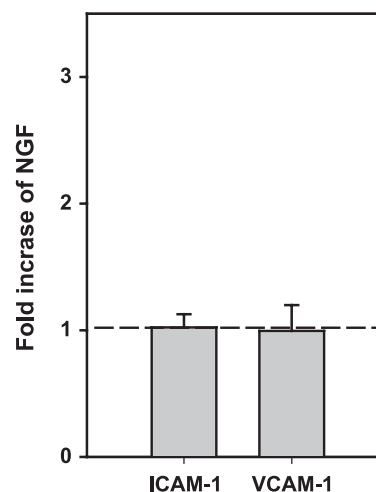


Fig. 4. Cross-linking the adhesion molecules ICAM-1 or VCAM-1 on activated astrocytes did not upregulate NGF production. In the figure, the control level (astrocytes only) is set to unity, indicated by the dashed horizontal line. Data are expressed as mean n -fold increase compared to control \pm S.D. from two independent experiments.

ICAM-1 is a costimulatory/adhesion molecule that is constitutively expressed on astrocytes and upregulated after treatment with IFN- γ (Aloisi et al., 1998). Astrocytes also express the adhesion molecule VCAM-1 when activated by IFN- γ (Lee and Benveniste, 1999). Cross-linking of ICAM-1 or VCAM-1 by adding the respective antibodies to activated astrocytes did not upregulate NGF production (Fig. 4).

4. Discussion

Activated T cells are capable of passing the blood–brain barrier (BBB), and thus infiltrate the brain parenchyma (Wekerle et al., 1986). Within the CNS, they can recognize peptides presented by the resident APCs via MHC class II, such as microglia and astrocytes (Shrikant and Benveniste, 1996), thereby contributing to a local immune response that might result in neuroinflammation. Therefore, it is of some interest to investigate, and possibly be able to differentiate, the manner in which Th1 and Th2 cells could affect NGF production in astrocytes. Another important point is the role of antigen-specific interactions.

In this study, we have demonstrated that NGF production is upregulated in astrocyte–T cell cocultures, and that this process is antigen-dependent. It is well documented that astrocytes produce NGF (Eddleston and Mucke, 1993). In addition, T cell lines have also been shown to produce NGF (Ehrhard et al., 1993; Santambrogio et al., 1994; Arredondo et al., 2001). However, the astrocytes in our experiments invariably produced a certain background level of NGF, which was significantly higher than the NGF levels in Th1 and Th2 cells cultured alone, or with APCs and antigen. Hence, we assume that

the detected upregulation of NGF in the cocultures of astrocytes and T cells is produced by the astrocytes rather than the T cells.

Previous studies have shown that Th2 cell-derived cytokines such as IL-4 and IL-10 upregulate NGF production in primary mouse astrocyte cultures, while the Th1 cell-derived cytokines IL-2 and IFN- γ do not. Furthermore, IFN- γ inhibits upregulation of NGF induced by IL-10 (Brodie, 1996; Brodie et al., 1998). We have shown that mechanism(s) other than cytokine production from activated Th1 and Th2 cells can upregulate NGF production in astrocyte–T cell cocultures. In our present study, this mechanism has been identified as antigen presentation. When neutralizing IL-10 and IL-4, no reduction in NGF production was observed. It is possible that the MHC class II–TCR interaction gives a much stronger signal than cytokine binding to its respective receptor. Moreover, the levels of IL-4 and IL-10 in our cultures did not reach as high as the levels that were added by Brodie (1996) and Brodie et al. (1998).

T cells that are not neuroantigen-specific play an important role in neuroinflammatory disease as they comprise the majority of T cells in inflammatory infiltrates (Steinman, 1996). Our finding of the involvement of TCR–MHC class II interactions in NGF upregulation does not exclude that these T cells may induce NGF upregulation, provided their specific antigen is presented by astrocytes. However, this is rather unlikely.

The finding that naïve T cells, irrespective of the presence of antigen, cannot induce upregulation of NGF could have been anticipated based on the fact that astrocytes fail to prime naïve T cells (Aloisi et al., 1999). Consequently, no sufficient signalling through the TCR–MHC class II pathway can induce an upregulation of NGF synthesis. Moreover, since it has been shown that only activated T cells can cross the BBB (Wekerle et al., 1986), interactions between naïve and resident CNS APCs are not relevant *in vivo*.

When direct contact between T cells and astrocytes was excluded by a membrane, we observed a reduced level of NGF in the coculture. As we demonstrated, this was neither due to an artefact induced by the membrane nor to a consumption of NGF by T cells. However, the cultivation medium was exchanged on the same day as an experiment with cocultures of astrocytes and T cells was started. Thus, the level of NGF in the cocultures with a membrane separating astrocytes and T cells never reached the general background level of NGF production in astrocytes. This phenomenon remains to be explained.

The NGF level in the cocultures with activated astrocytes and Th1 cells, when blocking MHC class II, was significantly higher than the control level. However, it was also significantly lower than in the cocultures without blocking MHC class II (Fig. 2B). This suggests that the MHC class II block was either incomplete, or that alternative pathways based on cellular interactions are involved. These could be facilitated by cognate MHC class II–TCR interactions, but not exclusively dependent on them. Since MHC class II

mismatch experiments demonstrated a slight but significant upregulation of NGF, other cellular interactions may also be involved. By cross-linking ICAM-1 and VCAM-1, we addressed this question by assuming that a ligation of adhesion molecules expressed on astrocytes could be responsible for the observed upregulation. This does not seem to be the case. However, we cannot fully exclude the involvement of other adhesion molecules that have not been described so far.

Although we assume that the upregulated NGF production derives from the astrocytes, it is possible that T cells are contributing to the observed upregulation as they possess the ability to produce NGF. To answer the question of which cell population is responsible for the observed NGF increase, RT-PCR analysis of the T cell fraction could be applied. Nevertheless, the outcome would remain inconclusive since it is impossible to separate the T cells from the astrocytes after coculturing without having a small contamination of astrocytes, which could give false-positive results for the T cells. Intracellular immunofluorescence staining was applied, but failed in our hands. Thus, experimental proof of either hypothesis is still lacking. However, either way, our data do show a potential neuroprotective mechanism unique to astrocytes, as professional APCs are unable to upregulate NGF production in T cells.

In different animal models of EAE, the importance of NGF in suppressing inflammatory responses is well established. We have found that astrocyte–T cell interactions upregulate NGF levels when astrocytes present antigen to T cells, irrespective of the T helper phenotype. This could constitute a feasible mechanism by which astrocytes can counteract the consequences of a T cell-mediated neuroinflammation in an efficient manner.

Acknowledgements

This study was supported by the Hertie-Stiftung (1.319.110/01/04) and the Bundesministerium für Bildung und Forschung (FKZ 01 ZZ 0108). The authors thank Prof. Asle Sudbø for critically reading the manuscript.

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