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RESEARCH ARTICLE

Astrocytic Factors Deactivate Antigen Presenting Cells that Invade the Central Nervous System

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We hypothesized that CNS tissue has the potential to deactivate invading monocytes/macrophages in order to maintain the immune privilege of the brain, and furthermore, that astrocytes are the cells that initiate monocyte/macrophage deactivation. To test this hypothesis, fluorescent prelabeled rat spleen macrophages with typical amoeboid morphology were transferred into organotypic hippocampal slice cultures (OHSCs), where they gradually developed a ramified morphology similar to the appearance of resting microglial cells. This morphological transformation also occurred if macrophages or monocytes were co-cultured with mixed glial cultures or with astrocytoma cells, and ramification was accompanied by reduced expression of adhesion molecules leukocyte function antigen (LFA)-1, intercellular adhesion molecule (ICAM)-1, and major histocompatibility complex (MHC)-class-II molecules. Moreover, treatment of macrophages with astrocyte culture supernatant effectively down-regulated the LPS-induced expression of adhesion- and MHC-class-II-molecules. Astrocyte supernatant-induced inhibition of adhesion and MHC-class-II-molecule expression was mimicked by transforming growth factor (TGF)-β1, furthermore, this inhibitory effect was diminished by simultaneous treatment with neutralizing anti-TGF-β-antibodies. In conclusion, our results suggest that astrocyte-derived, soluble factors that are present in the CNS microenvironment deactivate invading macrophages, thus contributing to the maintenance of CNS immune-privilege following impairment of blood-brain-barrier (BBB) integrity.

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Introduction

The CNS is considered an immune-privileged site, mainly due to the fact that immune responses elicited in the CNS are suppressed when compared to immune responses in the periphery (3, 40). Several mechanisms are thought to contribute to this immune-privilege: The most important mechanism seems to be represented by the BBB that limits influx of immunocompetent cells, antibodies (Abs) and components of the complement system into the CNS parenchyma (7, 22). Rigorous control over the activity of antigen-presenting cells (APCs) is probably an additional instrument of immune regulation, as is illustrated by the observation that microglial cells, the resident population of potential APCs in the CNS, show little or no constitutive expression of MHC-class-I and -II under physiological conditions (8, 24). Pathological conditions that interfere with the integrity of the BBB endanger this delicate regulation of immunological activity due to the subsequent increase in the number of infiltrating immunocompetent cells (30, 34). The immunological activity of infiltrating APCs inside the CNS must therefore be modulated to avoid excessive damage. It seems reasonable to assume that the ability of the CNS to deactivate invading APCs, i.e., monocytes/macrophages, represents a crucial mechanism to maintain or restore CNS immune-privilege.

The OHSC preserves not only neuronal but also non-neuronal aspects of the CNS, as studies on the morphology and distribution of astrocytes and oligodendrocytes have demonstrated (2, 5). We have recently shown that initially activated microglial cells inside OHSCs are subject to deactivating stimuli, as amoeboid microglial cells that express high levels of integrin adhesion molecules LFA-1 and very late antigen-4 (VLA-4) become

ramified and down-regulate integrin expression after 9 days *in vitro* [div](16, 17). From these findings we concluded that the OHSC provides a microenvironment that induces deactivation of previously activated microglial cells. Such deactivation of the CNS-resident population of APCs does not spontaneously occur in isolated microglial cultures, and it has been suggested that the CNS possesses a unique network of cytokines that supports the establishment and maintenance of this aspect of immune privilege (7, 29). A large number of investigations has recognized astrocytes as a major source of cytokines, among them several suppressive modulators of the immune response, e.g. IFN-β and TGF-β (6, 7, 9, 35, 36, 37).

All of these observations taken together, we hypothesized that – in analogy to the deactivation of microglial cells – activated macrophages that trespass the BBB should become deactivated when exposed to healthy CNS microenvironment. We therefore transferred rat spleen macrophages into OHSCs and investigated whether changes from amoeboid to ramified morphology, resembling that of resting microglial cells, took place. Moreover, if astrocytes were responsible for the deactivation of APCs, macrophages or blood monocytes should experience a similar morphological transformation and down-regulate their expression of immunological activation markers LFA-1, ICAM-1 and MHC-class-II when co-cultured with astrocytes or astrocytoma cells. Finally, if astrocytes secreted soluble factors, it should be possible to induce immunological deactivation of macrophages by treatment with astroctye culture supernatant. Consequently, flow cytometry analysis was used to assess the effects of astrocyte culture supernatant on ICAM-1-, LFA-1- and MHC-class-II-expression on activated rat spleen macrophages.

Materials and Methods

Culture of rat spleen macrophages. Primary cultures of rat spleen macrophages were prepared from spleens of Wistar rats. In brief, after ether anesthesia and decapitation of the animal the spleen was removed under sterile conditions. Following removal of the spleen capsule under the dissection microscope, the tissue was minced in Hanks' Ca^{2+}/Mg^{2+}-free balanced salts solution (HBSS, Gibco BRL Life Technologies, Eggenstein, FRG). The supernatant was diluted 1:1 with RPMI (Gibco), then shifted onto Ficoll separating solution (Seromed Biochrom, Berlin, FRG; density 1.077 g/ml) and centrifuged at 1000 x g for 20 min. at 4°C. The resulting layer of mononuclear cells was transferred into RPMI, washed and plated into cell culture wells of 24-well-plates (Falcon, Heidelberg, FRG) in RPMI with 10% fetal bovine serum (FBS), penicillin (100 U/ml; Sigma, Deisenhofen, FRG), streptomycin (0.1 mg/ml; Sigma), and glutamin (2%). Following a pre-plating period of 2 hrs under standard incubation conditions (37°C, 5% CO_2, 100% humidified atmosphere) the wells were repeatedly washed with RPMI to remove non-adherent lymphocytes. Rat spleen macrophage cultures were shown to be > 95% pure, as demonstrated by immunocytochemistry with monoclonal Abs against Mac-1 (OX-42, Camon/Serotec, Wiesbaden, FRG; dilution 1:100).

Rat glial cultures. Primary cultures of mixed cortical glial cells were prepared from neonatal Wistar rats (P0-P1) according to the method described previously (14). In brief, after removal of the meninges, cerebral cortices were dissociated in Ca^{2+}/Mg^{2+}-free HBSS, containing trypsin (4 mg/ml; Boehringer, Mannheim, FRG) and DNAse (0.5 mg/ml; Worthington, Bedford MA, USA). Cells were plated into 75 cm^2 tissue culture flasks (Falcon) containing Dulbecco's modified Eagle's medium (with 4.5 g/l Glucose, without sodium pyruvate; DMEM, Gibco), supplemented with 10% FBS, glutamine (2%), penicillin (100 U/ml) and streptomycin (0.1 mg/ml). This resulted in growth of a confluent astrocyte monolayer with adherent microglial cells. Further passaging by trypsinization enabled transfer of mixed glial cultures onto poly-L-lysine-coated glass coverslips (10μg/ml) placed on the bottom of cell culture wells. Cultures were grown until reaching confluency for further use in co-culture experiments with rat spleen macrophages. Microglia-depleted astroglial cultures were obtained by repeated mechanical dislocation of microglial cells and washing with HBSS. Threefold repetition of this procedure resulted in nearly pure astrocyte cultures, as verified by immunocytochemistry against glial fibrillary acidic protein (GFAP; $GFAP^+$-cells >98%). The supernatant from such cultures was centrifuged, filtered and frozen at -20°C until further use in experiments on adhesion molecule expression of rat spleen macrophages under the influence of astrocyte culture supernatant.

Preparation and maintenance of OHSCs. OHSCs were obtained by decapitating male Wistar rats (10 - 12 days p.n.) and aseptically removing a frontal slice of the caudal cerebrum. This slice was immersed in preparation medium and transversally sectioned at 350μm thickness on a sliding vibratome at 4°C. Medium for

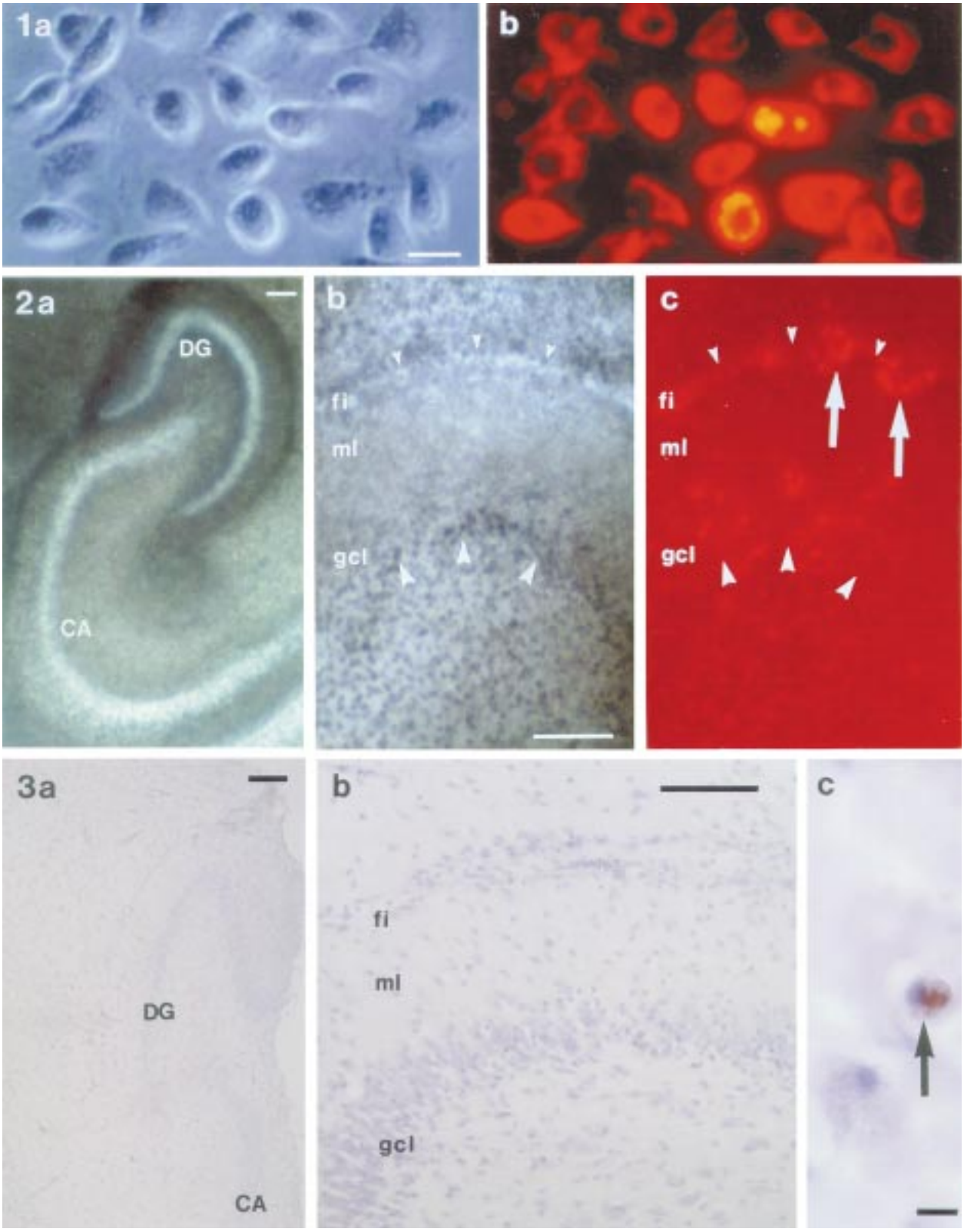
preparation (pH =7.35) consisted of minimum essential medium (MEM, Gibco), supplemented with glutamin (1%; Gibco). Slices were cultured on Falcon cell culture inserts (pore size 0.4μm) in 6 well plates and fed with 1 ml of medium according to standard protocols. The culture medium (pH=7.40) contained a basic mixture of MEM and HBSS in 2 : 1 proportions, and furthermore the following substances: normal horse serum 25% (NHS, Gibco), glutamine (2%), glucose (2.64 mg/ml; Braun, Melsungen, FRG), penicillin (100 U/ml), streptomycin (0.1 mg/ml), insulin (1μg/ml; Gibco) and vitamin C (0.8μg/ml; Sigma).

Human monocytes. Human monocytes were obtained from venous blood of healthy human donors by centrifugation on Ficoll separating solution (Seromed Biochrom; density 1.077 g/ml) at 1000 x g for 20 min. The resulting layer of peripheral blood mononuclear cells was removed, washed in RPMI and plated into cell culture wells of 24-well-plates. Further purification was analogous to the procedure described for rat spleen macrophages. Human monocyte cultures were shown to be > 95% pure, as demonstrated by immunocytochemistry with monoclonal Abs against Mac-1 (OX-42, Camon/Serotec; dilution 1:100).

Human astrocytoma cell line CCF-STTG1. The human astrocytoma cell line CCF-STTG1 has been established from a grade IV astrocytoma and expresses GFAP and MHC-class-II-antigens. It was purchased from the European Collection of Cell Cultures (Salisbury, UK) and cultivated in RPMI with 10% FBS, penicillin (100U/ml) and streptomycin (0.1mg/ml, Sigma). Propagation of this cell line in non-coated 75cm^2 cell culture flasks resulted in typical astrocytic morphology and the formation of confluent monolayers. For co-culture experiments, confluent cells were removed from the flasks by trypsinization (trypsin 0.05%/EDTA 0.02%), transferred onto poly-L-lysine-coated glass coverslips (10μg/ml) placed on the bottom of cell culture wells and grown until reaching confluency.

Labeling of rat spleen macrophages or human monocytes. Labeling of rat spleen macrophages or human monocytes was performed by incubation with culture medium containing Mini Ruby (10 kD dextran amine, rhodamin-conjugated and biotinylated; 20μg/ml; Molecular probes, Oregon OR, USA) for 72 hrs. This procedure resulted in stable fluorescent labeling of monocytes/macrophages and proved to be non-toxic. The fluorescent dye is actively incorporated into cells by pinocytosis and is usually found inside cytoplasmic granula (10). Due to its hydrophilic nature there was no leakage through the cellular membranes of intact, prelabeled cells and contamination of surrounding tissues did not occur. Prelabeled monocytes/macrophages were washed, detached from cell culture wells by incubation with Ca^{2+}/Mg^{2+}-free HBSS containing 5mM EDTA, centrifuged and washed again before transferring them onto OHSC surfaces, onto mixed glial cultures or onto CCF-STTG1-astrocytoma monolayers.

Macrophage invasion assay and immunohistochemistry. Fluorescence prelabeled rat spleen macrophages were transferred onto the surface of freshly prepared OHSCs. Observation of the invasion process was enabled by fluorescence microscopy of living OHSCs at all stages of the *in vitro*-culture period. After incubation for 3, 6, or 9 div slices were immersed in a fixative containing 4% formaldehyde, 0.1% glutaraldehyde and 15% picric acid. Before, between and after fixations, slices were thoroughly washed in 0.1 M phosphate buffer (PB; pH = 7.4). After immersion of slices in 0.8 M sucrose solution for 24 h 14 μm thin horizontal sections were prepared on a Jung cryostat 2800 frigocut-E (Cambridge Instruments, Nussloch, FRG). Sections were then thaw-mounted on gelatine coated glass slides and coverslipped with Immu-Mount (Shandon, Pittsburgh PA, USA). For labeling of macrophages and microglia, sections were washed 3 x 3 min. in PB and preincubated in NHS (diluted 1:10 in PB) for 30 min in a humid chamber, then the material was incubated overnight with *Griffonia simplicifolia* isolectin B_4 (GFS-B_4)-FITC (Sigma, diluted 1:40) in PB (0.1M, pH = 6.8) and 0.5% Triton X-100 and finally coverslipped with Immu-Mount. Following fluorescence microscopy, coverslips were removed in 0.1 M PB and the sections counterstained with hematoxyline, dehydrated (ethanol series of 70%, 80%, 96%, 100%, and twice in xylene) and finally coverslipped with Entellan. Only sections derived from cultures with completely intact hippocampal morphology were used for further analysis, ensuring that the occasionally observed effects of preferential migration of prelabeled macrophages towards the hippocampal fissure or towards the granule cell layer of the dentate gyrus was not due to neuronal or axonal degeneration in these areas. In a subset of experiments the rhodamin- and biotin-conjugated tracer Miniruby contained inside the prelabeled macrophages was converted into a DAB precipitate by incubating the sections with avidin and biotinylated horseradish peroxidase follow-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ing standard protocols (ABC Elite, Vectastain). The sections were examined with a BX-50 microscope (Olympus, Tokyo, Japan) and photographs were taken with a PM-20 camera system (Olympus). Fluorescence was evaluated with the following filters: FITC, excitation filter 470 - 490 nm, barrier filter 515 - 550 nm; rhodamine, excitation filter 520 - 550 nm, barrier filter 580 nm.

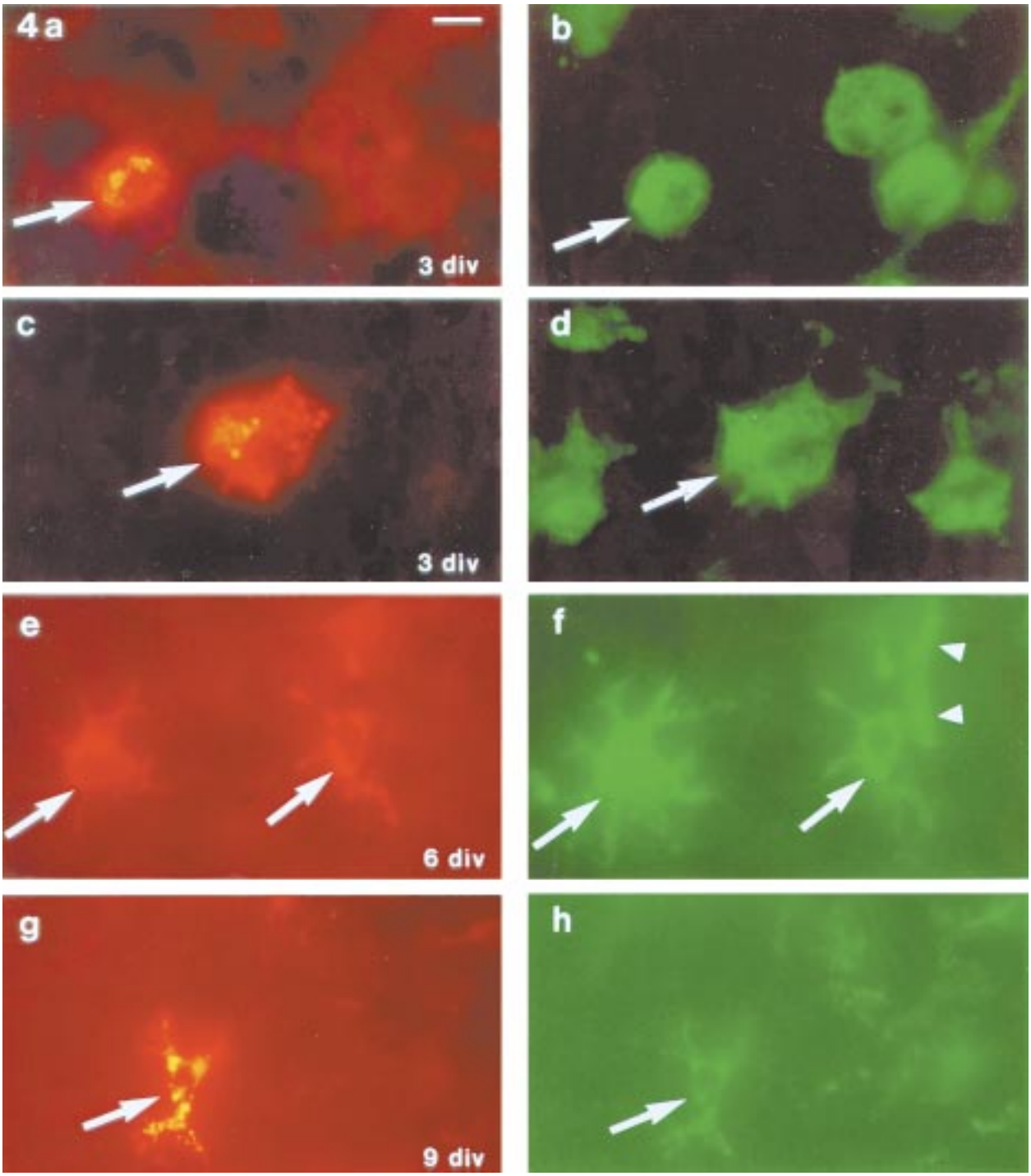
Co-culture of rat spleen macrophages with mixed glial cultures and co-culture of human monocytes with CCF-STTG1-cells and subsequent immunocytochemistry. Prelabeled rat spleen macrophages or human monocytes were transferred onto confluent mixed rat glial cultures or confluent CCF-STTG1 astrocytoma monolayers and incubated under standard conditions in DMEM with 10% FBS, penicillin/streptomycin (100 U/ml; 0.1 mg/ml) and glutamine (1%). Daily observation using fluorescence microscopy was performed until fixation of co-cultures after variable *in vitro*-periods. Co-cultures were fixed with 4% formaldehyde, 0.1% glutaraldehyde and 15% picric acid and immunocytochemistry using the following Abs was performed: Coverslips were washed and preincubated in NHS (diluted 1:10 in PB) for 30 min., followed by either anti-rat-CD11a (LFA1α, clone WT.1)-Ab, anti-rat-CD18 (LFA-1β, clone WT.3)-Ab, anti-rat-CD54 (ICAM-1, clone A29)-Ab, or anti-rat-MHC-II-(RT1.B, clone P7/7)-Ab for rat spleen macrophages, or either anti-human-CD11a (LFA1α, clone DF1524)-Ab or anti-human-CD54 (ICAM-1, clone 6.5B5)-Ab for human monocytes, all diluted 1:200 in 0.1M PB, Triton X-100 (0.5%) and NHS (0.1%), lasting overnight. Material was washed again and incubated for 2 hrs in goat-anti-mouse-IgG-DTAF (Dianova, Hamburg, Germany, diluted 1:100 in 0.1M PB) with 0.5% Triton X-100. For astrocyte labeling, coverslips were incubated overnight in 0.1M PB with rabbit-anti-GFAP-Ab (diluted 1:1,000; Boehringer) and Triton X-100 (0.5%) and NHS (0.1%). Finally, coverslips were washed, mounted on glass slides using Immu-Mount (Shandon, Pittsburgh PA, USA) and examined on the microscope described above. The hydrophilic tracer Miniruby was present in the cytoplasm, sometimes inside small cytoplasmic vesicles, but was never observed inside the nucleus. Rhodamine fluorescence following Miniruby-incubation thus gave an outline of the macrophage cytoplasm with lack of fluorescence in the nuclear area.

FACS analysis of the expression of adhesion molecules and MHC-class-II-molecules on rat spleen macrophages. Isolated rat spleen macrophages were treated with microglia-depleted astrocyte culture supernatant (30%), or LPS (500ng/ml), or TGF-β1 (0.5 ng/ml), or neutralizing anti-TGF-β-Abs (20μg/ml; Sigma), or with combinations of LPS and astrocyte culture supernatant or of LPS with astrocyte culture supernatant and with anti-TGF-β-Abs. For experiments using a combination of LPS, astrocyte supernatant and neutralizing anti-TGF-β-Abs, the neutralizing Abs were incubated with the astrocyte supernatant at 37°C for 1 h before addition to LPS-containing medium. Controls were incubated in normal culture medium. After 72 hrs incubation in culture wells of 24-well-plates the cells were carefully detached by gentle handling with a glass pipette in $HBSS^-$ containing 5 mM EDTA and then transferred into 5 ml FACS tubes (Falcon). After centrifugation (100 x g, 4 min., 4°C) cell pellets were fixed with a fixative containing 4% paraformaldehyde, 15% of a saturated solution of picric acid and 0.1% glutaraldehyde for 8 min. on ice. After repeated washing with PBS containing 1% bovine serum albumin (BSA) cells were incubated either with mouse anti-rat LFA-1α-Abs, mouse anti-rat MHC-class-II-Abs or mouse anti-

Figure 1. (Opposing page) Rat spleen macrophages in vitro. **a)** Phase contrast microscopy of isolated rat spleen macrophages in vitro, displaying typical amoeboid morphology (scale bar: 10μm). **b)** Corresponding area to **a)**, showing fluorescence microscopy of rat spleen macrophages labeled with the fluorescent dye Mini Ruby.

Figure 2. (Opposing page) Invasion of prelabeled rat spleen macrophages into organotypic hippocampal slice cultures in vitro. **a)** Overview over organotypic hippocampal slice culture, showing the cornu ammonis (*CA*) and the dentate gyrus (*DG*). **b)** Transmission and **c)** fluorescence microscopy of identical areas in the dentate gyrus, showing the hippocampal fissure (*fi*, small arrowheads), the molecular layers (*ml*), and the granule cell layer (*gcl*, large arrowheads). Fluorescent rat spleen macrophages are visible in **c)**, and accumulation of macrophages along the hippocampal fissure (arrows) and inside the granule cell layer occurs (scale bars: 100μm).

Figure 3. (Opposing page) Organotypic hippocampal slice culture after fixation and sectioning. **a)** Overview over the entire dentate gyrus (*DG*), the hilar region and the CA3-portion of the cornu ammonis (*CA*) inside the intermediate layer of an OHSC following fixation, sectioning and hematoxylin staining after 9 div (scale bar: 100μm). **b)** Detail of the dentate gyrus with the granule cell layer (*gcl*), the molecular layers (*ml*) and the hippocampal fissure (*fi*), showing intact cytoarchitecture of this region after 9 div (scale bar: 100μm). **c)** High power magnification of a prelabeled rat spleen macrophage that has invaded the intermediate layer of an OHSC; following DAB-conversion of the biotinylated tracer Mini Ruby (brown) and hematoxylin-counterstain (blue; scale bar: 10μm).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rat ICAM-1-Abs, all diluted 1:200 in PBS with 0.5% Triton X-100, for 2 hrs on ice, washed three times with PBS containing 1% BSA, and then incubated with goat-anti-mouse-Ig-FITC (Becton Dickinson, Heidelberg, Germany; undiluted) for 30 min. on ice. After repeated washing with PBS containing 1% BSA, 5,000 cells from each sample were analyzed on a FACStrak (Becton Dickinson), uniformly gated and the mean fluorescence intensity (FL1-channel) of all gated cells detected. The expression of MHC-class-II-, ICAM-1-, or LFA-1α was calculated by the equation x-Fold Increase= $FL1_{Experiment}/FL1_{Control}$. Experiments were repeated 6 to 12fold and statistical significance was investigated using the Wilcoxon-matched-pairs test.

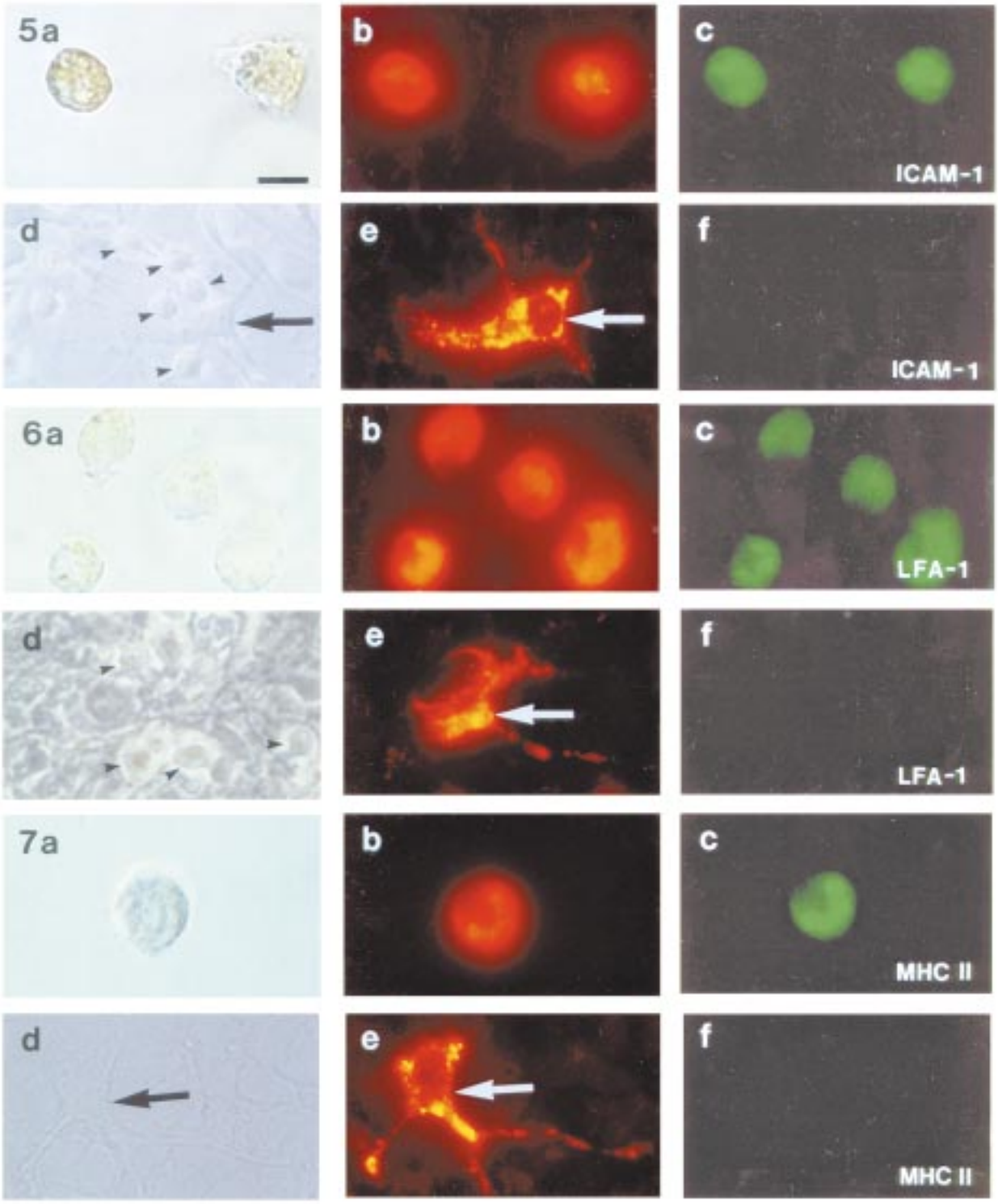
Results

Amoeboid rat spleen macrophages migrate into OHSCs and ramify. Rat spleen macrophages in single cell culture were incubated with the fluorescent dye Mini Ruby, resulting in rapid and stable fluorescent labeling of all macrophages (Fig. 1). Prelabeled macrophages were then transferred onto OHSCs and in vitro-fluorescence microscopy revealed migration of prelabeled macrophages into the intermediate layer of OHSCs (Fig. 2). Migration of prelabeled macrophages seemed to be preferentially directed towards certain areas of OHSCs: In the hippocampus it was observed that prelabeled macrophages seemed to accumulate in the region of the hippocampal fissure and along the granule cell layer of the dentate gyrus (Fig. 2c). The migration of prelabeled macrophages into the intermediate layer predominantly occurred during the first 3 days in vitro (div). During this period, all observed macrophages displayed amoeboid morphology, sometimes with short, single pseudopodia. After 6 div, invading macrophages inside the intermediate OHSC layer began to develop multiple, relatively short cytoplasmic processes, whereas macrophages that had remained on OHSC surfaces persistently displayed amoeboid morphology. Further development of cytoplasmic processes could be observed up to 9 div, however, precise in vitro-observations of prelabeled cells inside the intermediate layers of OHSCs were difficult.

Fixation and sectioning of OHSCs enabled separate microscopic examination of superficial, intermediate and bottom layers of hippocampal slice cultures. For further analysis, only OHSCs with intact hippocampal morphology, as verified by hematoxyline counterstains, were selected (Fig. 3). DAB-conversion of the biotinylated tracer Mini Ruby resulted in brown staining of prelabeled macrophages that had invaded the intermediate layer of OHSCs. The transformation of initially amoeboid macrophages to cells with a relatively compact cytoplasm and delicate, branched cytoplasmic processes was observed in most macrophages that had invaded the intermediate layer: After 3 div amoeboid prelabeled macrophages were observed in the superficial and intermediate layers of OHSCs (Fig. 4a). At this stage some macrophages already displayed single or multiple filopodial processes, indicating the beginning of the process of ramification (Fig. 4c). After 6 div, most prelabeled macrophages inside the intermediate layer had developed multiple cytoplasmic processes, whereas prelabeled macrophages that had remained in the superficial layer showed no such signs of morphological transformation (Fig. 4e). After 9 div, prelabeled macrophages resident in the intermediate layer showed multiple, delicately branched cytoplasmic processes (Fig. 4g).

Double immunofluorescence using GFS-B_4 visualized prelabeled macrophages and endogenous microglia. Prelabeled cells were always double-labeled by GFS-B_4, confirming that the dye was confined to the macrophages that had been initially labeled in the single cell culture, and that no leakage of the dye to surrounding astrocytes, neurons or oligodendrocytes had occurred (Fig. 4b, d, f, h). Morphological changes of

Figure 4. (Opposing page) Morphological transformation of spleen macrophages inside organotypic hippocampal slice cultures. Rhodamin **(a, c, e, g)**- and FITC **(b, d, f, h)**-fluorescence microscopy of sections from the intermediate layer of OHSCs, showing fluorescent prelabeled spleen macrophages and GFS-B_4-FITC-counterstains. **a)** Amoeboid prelabeled macrophage after 3 div (arrow), **b)** identical section to a) showing GFS-B_4-double-staining of the prelabeled macrophage (arrow). Several GFS-B_4-stained but non-prelabeled amoeboid microglia are also visible. **c)** Amoeboid prelabeled macrophage (arrow) after 3 div displaying several filopodial processes as initial signs of further ramification. Note the organization of the fluorescent tracer Mini Ruby inside cytoplasmic granulae and the absence of nuclear staining. **d)** Corresponding section to **c)** showing simultaneous GFS-B_4-staining of the prelabeled macrophage (arrow) and several GFS-B_4-stained, but non-prelabeled amoeboid microglial cells. **e)** Prelabeled macrophages after 6 div (arrows) with development of relatively coarse cytoplasmic processes without secondary branches. **f)** Corresponding FITC-fluorescence-image to **e)** showing simultaneous GFS-B_4-staining of prelabeled macrophages (arrows). Additional GFS-B_4-staining of cerebrovascular endothelia (arrowheads). **g)** Prelabeled macrophage after 9 div (arrow) showing branched cytoplasmic processes of fine caliber. **h)** Simultaneous GFS-B_4-staining of the prelabeled macrophage (arrow) shown in **g)** and additional staining of ramified microglia. Scale bar: 10μm for all pictures.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GFS-B_4-labeled microglial cells paralleled those observed in prelabeled macrophages: Early in vitro-stages showed amoeboid microglia in all layers of OHSCs, whereas cultivation for 6 or 9 div resulted in ramification of microglial cells located in the intermediate layer. The ramified microglia inside the intermediate layer were morphologically indistinguishable from ramified prelabeled macrophages. Microglia in superficial and bottom layers continued to display amoeboid morphology throughout the entire period of in vitro-culture.

Co-culture of rat spleen macrophages with mixed glial cultures induces ramification and down-regulation of adhesion molecule expression and MHC-class-II-expression. Co-culture of prelabeled rat spleen macrophages with mixed glial cultures was performed to investigate whether glial cells alone were able to induce the transformation of macrophages that had been observed inside OHSCs. It was observed that a morphological transformation process of prelabeled macrophages that resembled the transformation seen in OHSCs did also occur in co-culture with mixed glial cultures (Figs. 5d-f, 6d-f, 7d-f), whereas control macrophages in single culture retained their amoeboid morphology and did not down-regulate the expression of ICAM-1, LFA-1 or MHC-class-II (Figs. 5a-c, 6a-c, 7a-c). Amoeboid macrophages, observed at the beginning of the co-culture period, were gradually transformed into ramified macrophages with multiple cytoplasmic processes and smaller somata. Most cytoplasmic processes developed secondary branches of fine calibers (Figs. 5e, 6e, 7e). These transformed cells interacted very closely with the underlying astrocytic monolayer and macrophage processes sometimes even seemed to intercalate between astrocytes. There was not such close association of prelabeled macrophages with the microglial cells that were also present in the co-culture. The process of morphological transformation of prelabeled macrophages was observed during an in vitro-culture period of 9 days, i.e., kinetics of morphological transformation in mixed glial cultures resembled those registered in OHSCs. The morphological transformation of prelabeled macrophages in mixed glial cultures was accompanied by down-regulation of the expression of adhesion molecules ICAM-1 and LFA-1 and of MHC-class-II-molecules, as verified by immunocytochemistry. Amoeboid macrophages, as observed in an early stage of co-culture or in isolated macrophage culture, were intensely stained by Abs directed against ICAM-1 (Fig. 5c), LFA-1 (Fig. 6c), and MHC-class-II (Fig. 7c), whereas morphologically transformed macrophages, as observed after 9 days in co-culture with glial cells,

Figure 5. (Opposing page) Morphological transformation and down-regulation of ICAM-1-expression of rat spleen macrophages following co-culture with mixed glial cultures. **a-c)** Control experiment with fluorescent prelabeled macrophages in isolated culture after 9 div. **a)** Phase contrast microscopy of two amoeboid macrophages, **b)** rhodamin-fluorescence of these two prelabeled macrophages, **c)** FITC-fluorescence microscopy following staining for ICAM-1. The macrophages still display spherical or amoeboid morphology and ICAM-1 is expressed. **d-f)** Macrophage co-culture with mixed glial cells after 9 div. **d)** Phase contrast microscopy showing astrocyte monolayer, several amoeboid microglial cells (arrowheads) and macrophage (arrow). **e)** The macrophage is identified by rhodamin-fluorescence microscopy, visualizing the prelabeled cell with its delicate cytoplasmic processes following morphological transformation. **f)** FITC-fluorescence microscopy following immunostaining for ICAM-1 does not result in detectable staining of the prelabeled cell shown in **d)** and **e)**. Scale bar: 10μm for all figures on this page.

Figure 6. (Opposing page) Morphological transformation and down-regulation of LFA-1-expression of rat spleen macrophages following co-culture with mixed glial cultures. **a-c)** Control experiment showing fluorescent prelabeled macrophages after 9 div without co-culture with mixed glial cells. **a)** Phase contrast microscopy of four amoeboid macrophages, **b)** shows rhodamin-fluorescence of the prelabeled macrophages, **c)** FITC-fluorescence microscopy after staining for LFA-1. LFA-1 is expressed on the prelabeled macrophages and no morphological transformation has occurred. **d-f)** Macrophage co-culture with mixed glial cells after 9 div. **d)** Phase contrast microscopy shows the astrocyte monolayer with several amoeboid microglial cells on its surface (arrowheads). **e)** The prelabeled spleen macrophage, visualized by rhodamin-fluorescence microscopy, has been morphologically transformed, now showing cytoplasmic processes of fine caliber and secondary branches. **f)** Immunostaining for LFA-1 and subsequent FITC-fluorescence microscopy does not result in fluorescence of the prelabeled macrophage that is shown in **d)** and **e)**.

Figure 7. (Opposing page) Morphological transformation and down-regulation of MHC-class-II-expression of rat spleen macrophages following co-culture with mixed glial cultures. **a-c)** Control experiment with fluorescent prelabeled spleen macrophages in isolated culture after 9 div. **a)** Phase contrast microscopy of spleen macrophage, **b)** rhodamin-fluorescence microscopy of the prelabeled cell in **a)**, **c)** FITC-fluorescence microscopy following staining for MHC-class-II shows expression of the antigen on the prelabeled macrophage. The macrophage is still spherical and has not experienced morphological transformation. **d-f)** Co-culture of spleen macrophages with mixed glial cells after 9 div. **d)** Phase contrast microscopy shows a spleen macrophage that is embedded in the astrocyte monolayer (arrow). **e)** The prelabeled macrophage vaguely seen in **d)** is visualized by rhodamin-fluorescence microscopy, showing a prelabeled cell following morphological transformation, now possessing cytoplasmic processes. **f)** FITC-fluorescence microscopy following immunostaining for MHC-class-II does not result in detectable staining of the prelabeled cell shown in **d)** and **e)**.

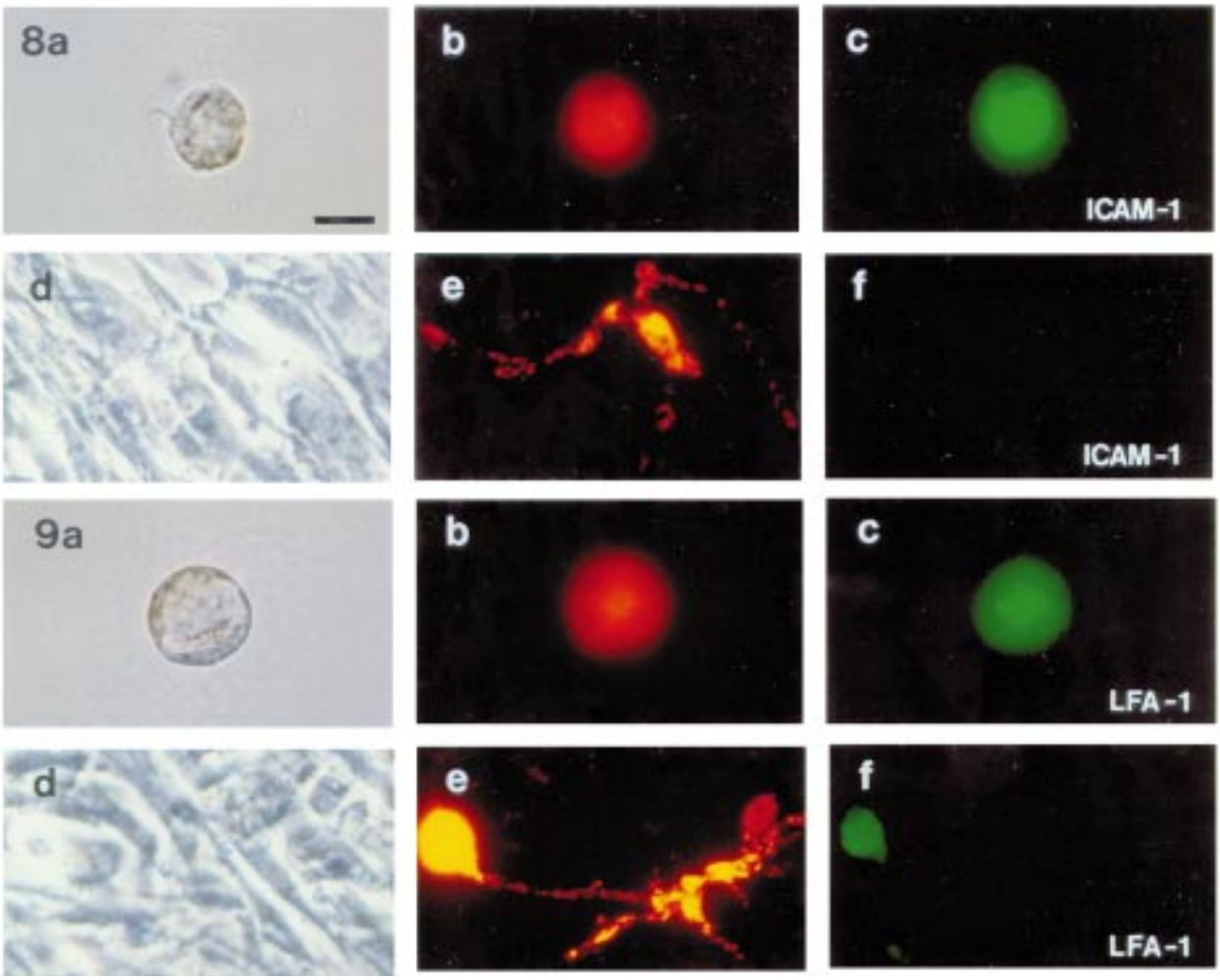

Figure 8. Co-culture of human monocytes with CCF-STTG1-astrocytoma cells results in morphological transformation and down-regulation of ICAM-1-expression. **a-c)** Control experiment with isolated human monocytes. **a)** Phase contrast microscopy showing spherical monocyte without cytoplasmic processes. **b)** Rhodamin-fluorescence microscopy showing fluorescence of the prelabeled monocyte shown in **a)**. **c)** FITC-fluorescence microscopy after immunostaining for ICAM-1, showing immunolabeling of the monocyte shown in **a)** and **b)**. **d-f)** Co-culture of human monocytes with CCF-STTG1-astrocytoma cells. Co-culture results in ramification of formerly amoeboid monocytes and in down-regulation of ICAM-1-expression. **d)** Phase contrast microscopy shows the astrocytic monolayer and one of the cytoplasmic processes of a monocyte. **e)** Rhodamin-fluorescence microscopy visualizes the entire prelabeled monocyte with its delicate cytoplasmic processes. **f)** FITC-fluorescence microscopy following application of Abs directed against ICAM-1 does not result in immunofluorescence of the prelabeled monocyte. Scale bar: 10μm for all figures on this page.

Figure 9. Co-culture of human monocytes with CCF-STTG1-Astrocytoma cells results in morphological transformation and down-regulation of LFA-1-expression. **a-c)** Control experiment with isolated human monocytes. **a)** The phase contrast photograph shows a spherical monocyte without cytoplasmic processes. **b)** Rhodamin-fluorescence microscopy visualizes fluorescence of the prelabeled monocyte shown in **a)**. **c)** FITC-fluorescence microscopy after immunostaining for LFA-1 results in immunolabeling of the monocyte shown in **a)** and **b)**. **d-f)** Co-culture of human monocytes with CCF-STTG1-astrocytoma cells induces ramification of amoeboid monocytes and down-regulation of LFA-1-expression. **d)** Phase contrast microscopy shows the CCF-STTG1-astrocytoma monolayer and one amoeboid monocyte (arrowhead) on the monolayer surface. **e)** Rhodamin-fluorescence microscopy of the area shown in **d)**, visualizing two prelabeled monocytes, one amoeboid (arrowhead) and one ramified (arrow). **f)** FITC-fluorescence following immunostaining for LFA-1. The amoeboid monocyte shows immunofluorescence, whereas the ramified cell is not immunoreactive.

showed no or very low immunoreactivity for these molecules (Figs. 5f, 6f, 7f). Astrocytes and ramified microglial cells did not express detectable levels of LFA-1, ICAM-1 or MHC-class-II.

Co-culture of human monocytes with CCF-STTG1 astrocytoma cells results in ramification and reduced adhesion molecule expression. The co-culture of human monocytes with CCF-STTG1-astrocytoma cells enabled the investigation of the isolated interaction of monocytes with astrocytes, eliminating the potential influence of either activated or resting microglial cells that had been present in the mixed glial cultures. Again, prelabeled human monocytes in isolated culture showed spherical shape or sometimes developed an amoeboid appearance (Figs. 8a,b; 9a,b), whereas co-culture of prelabeled monocytes with CCF-STTG1-astrocytoma cells resulted in the same morphological transformation that had been observed in rat spleen macrophages that were co-cultured with mixed glial cultures: From being spherical or sometimes amoeboid, human monocytes developed branched cytoplasmic processes and a smaller soma when co-cultured with CCF-STTG1-astrocytoma cells (Figs. 8e, 9e). This transformation was initiated after 3 div and was most often concluded after 9 div. Again, prelabeled monocytes were seen to develop close physical interaction with CCF-STTG1-astrocytoma cells: Spherical monocytes, observed at the beginning of the co-culture period, were readily visible in phase-contrast microscopy. Further in vitro-cultivation resulted in the gradual disappearance of such spherical cells, and the developing ramified, prelabeled macrophages were only detectable using fluorescence microscopy.

Morphological transformation of human monocytes in co-culture with CCF-STTG1-astrocytoma cells was accompanied by reduced expression of adhesion molecules LFA-1 and ICAM-1. It was observed that spherical or amoeboid monocytes, as seen in single monocyte culture or at the beginning of the co-culture period, showed intensive fluorescence after staining for ICAM-1 (Fig. 8c) and LFA-1 (Fig. 9c) . Prelabeled cells with branched cytoplasmic processes and smaller somata that developed after several days of co-culture with CCF-STTG1-astrocytoma cells showed very low or undetectable levels of ICAM-1- (Fig. 8f) or LFA-1-expression (Fig. 9f).

Treatment of rat spleen macrophages with astrocyte supernatant or with TGF-β1 induces down-regulation of adhesion molecules and MHC-class-II. To assess whether the down-regulation of adhesion molecules LFA-1α and ICAM-1 and MHC-class-II on rat spleen macrophages in co-culture with mixed glial cells was induced by astrocyte-derived soluble factors, rat spleen macrophages were treated with the supernatant from rat astroglial cultures, and expression of LFA-1α, ICAM-1 and MHC-class-II was quantitatively assessed by FACS analysis (Fig. 10). Stimulation of rat spleen macrophages with LPS resulted in an increase in MHC-class-II-expression ($p < 0.05$; Fig. 10a), however, treatment with rat astrocyte supernatant or with TGF-β1 after stimulation with LPS inhibited up-regulation of MHC-class-II (LPS vs. [LPS + astrocyte supernatant]: $p < 0.05$). When neutralizing anti-TGF-β-Abs where added to macrophages that were stimulated with LPS and treated with astrocyte culture supernatant, the inhibition of MHC-class-II-expression seemed to be weakened (control vs. [LPS + astrocyte culture supernatant + anti-TGF-β-Abs]: $p < 0.05$). Expression of MHC-class-II on rat spleen macrophages proved to be highly sensitive to treatment with TGF-β1, as LPS-induced up-regulation of MHC-class-II was partly inhibited by incubation with TGF-β1 (LPS vs. LPS + TGF-β1: $p < 0.05$).

Expression of LFA-1α on rat spleen macrophages treated with LPS was upregulated when compared to controls (controls vs. LPS: $p < 0.05$; Fig. 10b), although the expression of this integrin adhesion molecule was less pronounced than expression of MHC-class-II or ICAM-1. Induction of LFA-1α-expression by LPS was inhibited when rat spleen macrophages were simultaneously treated with astrocyte supernatant (LPS vs. [LPS + astrocyte supernatant]: $p < 0.05$), moreover, it was observed that rat spleen macrophages treated with LPS and TGF-β1 displayed lower levels of LFA-1-expression than cultures stimulated with LPS. Anti-TGF-β-treatment of cells that were stimulated with LPS and incubated with astrocyte culture supernatant seemed to weaken the inhibition that was exerted by the supernatant, although large variations in various experiments prevented these data from reaching the 95% level of significance when direct comparison of LPS + astrocyte culture supernatant with LPS + astrocyte culture supernatant + anti-TGF-β-Abs was performed.

Treatment of spleen macrophages with LPS induced a fairly robust increase in ICAM-1-expression (controls vs. LPS: $p < 0.05$; Fig. 10c), and this increase was similarly prevented by simultaneous treatment with astrocyte culture supernatant or with TGF-β1 (LPS vs. [LPS

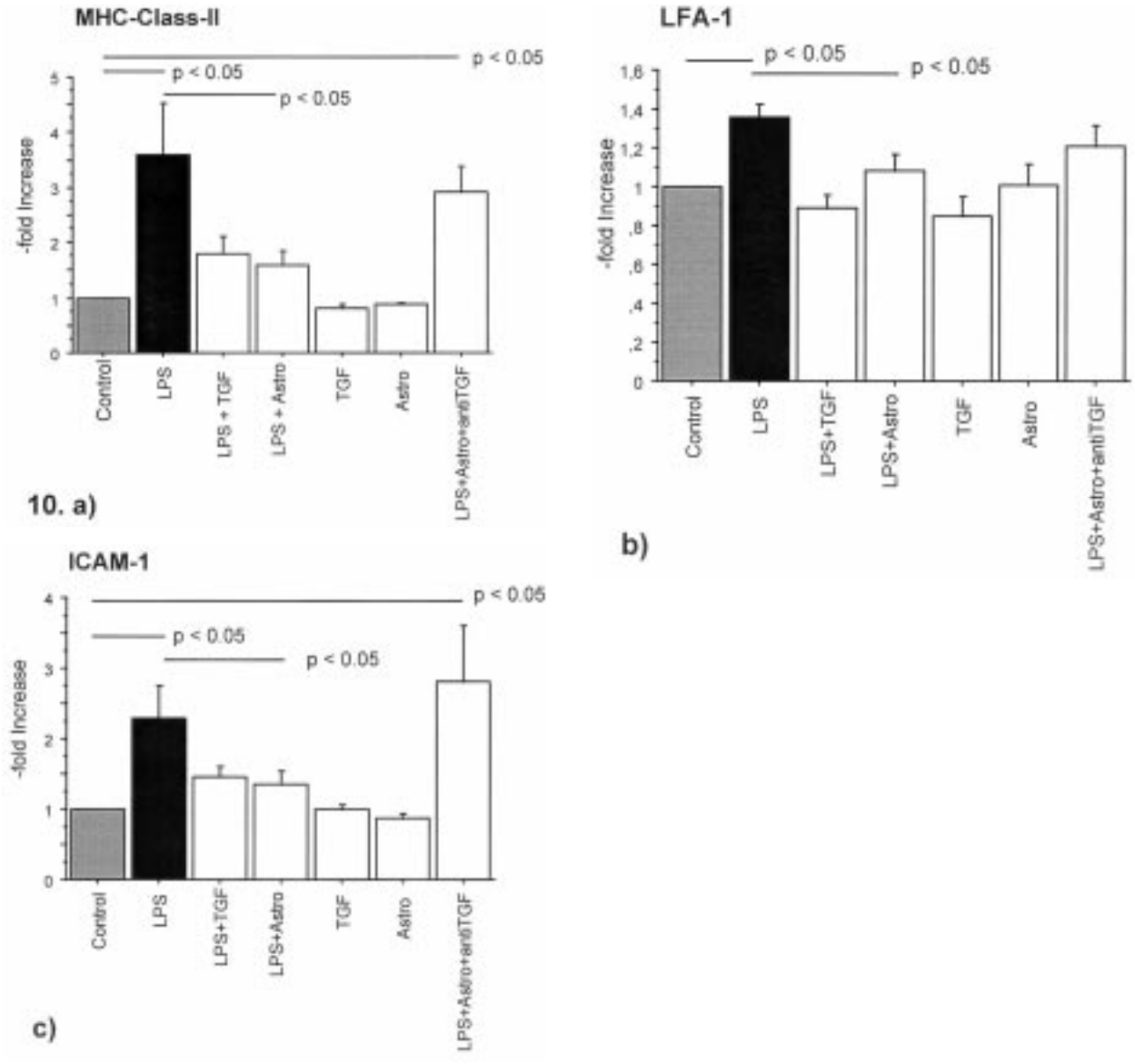

Figure 10. Treatment of stimulated rat spleen macrophages with astrocyte supernatant results in down-regulation of surface MHC-class-II, LFA-1, and ICAM-1. **a)** Expression of MHC-class-II following treatment of rat spleen macrophages with LPS, astrocyte culture supernatant (Astro), TGF-β1, neutralizing anti-TGF-β-Abs, or combinations of these substances. Astrocyte culture supernatant alone or TGF-β1 alone did not significantly alter the expression of MHC-class-II but prevented the upregulation of MHC-class-II on LPS-stimulated macrophages. Cells that were LPS-stimulated and treated with astrocyte culture supernatant again expressed increased levels of MHC-class-II when neutralizing anti-TGF-β-Abs were added. **b)** Histogram showing LFA-1-expression on rat spleen macrophages following treatment with LPS, astrocyte culture supernatant (Astro), TGF-β1, neutralizing anti-TGF-β-Abs, or combinations of these substances. The LPS-induced increase in LFA-1 was suppressed by treatment with TGF-β1 or with astrocyte culture supernatant. **c)** Summary of experiments on ICAM-expression, demonstrating that treatment of LPS-stimulated macrophages with astrocyte supernatant (Astro) or with TGF-β1 resulted in lower levels of ICAM-1-expression, whereas these substances alone had no significant effect. Neutralizing Abs directed against TGF-β seemed to disinhibit LPS-induced ICAM-1-expression on cells that were treated with astrocyte culture supernatant. All bars show mean values from 6 to 12 experiments with standard errors of mean.

+ astrocyte culture supernatant: $p < 0.05$). The impediment of LPS-induced ICAM-1-upregulation that was brought about by astrocyte culture supernatant could be abrogated by using neutralizing anti-TGF-β-Abs.

Discussion

Microglial cells, the resident APCs in the CNS, are easily activated in nearly all brain pathologies, and subsequent deactivation is also known to take place *in vivo* (4, 12, 13, 15, 19, 24, 27, 31, 38, 42, 43). This deactivation is characterized by restoration of ramified morphology and down-regulation of adhesion molecule- and MHC-class-II-expression, and immunological deactivation is thought to be the consequence of a unique network of cytokines inside the CNS, some of them with known antiinflammatory capacity (32, 33). Accordingly, our recent studies have shown that previously activated microglial cells are deactivated when exposed to the microenvironment provided by OHSCs (16, 17).

In the present study we hypothesized *a)* that the CNS microenvironment has the capacity to deactivate not only microglial cells but also activated macrophages or blood monocytes that trespass a disrupted BBB, and *b)* that astrocyte-derived factors are responsible for the deactivation of APCs. We therefore transferred rat spleen macrophages onto OHSCs, finding that amoeboid, spleen-derived macrophages invading the intermediate layer of OHSCs developed a ramified morphology very similar to that observed in resting microglial cells. Furthermore, spleen macrophages or blood monocytes were co-cultured with astrocytes, where ramification and down-regulation of the expression of LFA-1, ICAM-1 and MHC-class-II occurred. Finally, down-regulation of these immunoeffector molecules was also observed after treatment of spleen macrophages with astrocyte culture supernatant, indicating that soluble, astrocyte-derived factors were indeed responsible for the deactivating stimuli that seem to be present inside an organotypic CNS microenvironment.

The intermediate layer of OHSCs is considered to be organotypic not only from a neuronal but also from a glial point of view (2, 5, 17, 16). We therefore used the hippocampal slice culture as an experimental model to investigate the behavior of spleen macrophages inside the CNS-microenvironment, although such experiments are difficult to compare with the *in vivo*-situation or with *in vitro* co-culture assays. It has to be taken into account that spleen macrophages that have penetrated the surface of slice cultures can differ considerably from blood monocytes that have entered the brain via the BBB (30). Furthermore, it has to be emphasized that the ramification of macrophages does not necessarily correlate with immunological deactivation, although morphological changes in microglia are commonly associated with their immunological state of activation (42). Nevertheless, it was observed that invading macrophages inside an organotypic CNS microenvironment become morphologically indistinguishable from microglial cells, and we found that the expression of immunoeffector molecules is downregulated on macrophages or monocytes that ramify in co-culture with astrocytes. Taken together, this indicates that the CNS microenvironment controls not only endogenous, but also exogenous APCs that have trespassed the BBB, and that changes in morphology correlate with changes in immunological phenotype.

Preferential migration of prelabeled macrophages towards the hippocampal fissure and the granule cell layer was occasionally observed in our experiments and can be seen in Fig. 2c. It is well known that microglial cells are attracted towards sites of injured or degenerating neurons or axonal structures (26, 28, 42), and the same could apply to the spleen macrophages that migrated towards the hippocampal fissure in our recent experiments. Although no experimental lesion of OHSCs was performed this preferential migration could be explained by the fact that some of the very sensitive perforant path axons that span the hippocampal fissure are damaged by the preparation procedure itself, and this could induce subsequent migration of some macrophages towards this minimally damaged region. However, fixation, sectioning and counterstaining of our cultures with hematoxylin clearly confirmed that the structures of the dentate gyrus and the hippocampus proper of the OHSCs were intact (Fig. 3).

The finding of morphological transformation of spleen macrophages in co-culture with mixed glial cultures supports the hypothesis that glial cells provide the stimuli that induce ramification. Moreover, co-culture of human monocytes with CCF-STTG1 astrocytoma monolayers showed identical results, thus indicating that astrocytes but not activated or resting microglial cells, present in the mixed rat glial cultures, are necessary for the induction of morphological transformation. Our observations are in accordance with experiments on the co-culture of either microglial cells or macrophages with astrocytes or treatment with astrocyte culture supernatant: Liu et al. presented evidence that indicates a prominent role of M-CSF in the process of astrocyte-induced deactivation, and other experiments have shown that GM-CSF or antioxidants are potent inductors of microglial ramification (11, 18, 23, 39, 47). Further to

these soluble factors it is also possible that insoluble membrane components of astrocytes are responsible for the observed processes of ramification, as ramification of microglial cells on fixed astrocyte monolayers has been reported (45). It will be very important to determine whether transformations in the phenotype of macrophages are astrocyte-specific, or whether other cell-types can induce similar changes. The findings of Wilms et al. suggest that epithelial but not mesenchymal cells have the capacity to induce deactivation of macrophages, as ramification of rat macrophages was observed following co-culture with rat hepatoma cells MH1C1 or with the renal epithelial cell line NRK-52E, whereas co-culturing with fibroblasts only induced slight morphological changes (47).

As was mentioned above it has to be emphasized that morphological transformations of macrophages or microglial cells need not necessarily correlate with processes of immunological activation or deactivation, and we consequently investigated whether the expression of adhesion molecules LFA-1 and ICAM-1 and MHC-class-II-molecules is also influenced by co-culture with astrocytes or treatment with astrocyte supernatant. Apparently, co-culture of spleen macrophages or blood monocytes with astrocytes induced down-regulation of LFA-1, ICAM-1 and MHC-class-II, and this down-regulation also occurred after treatment of LPS-stimulated spleen macrophages with astrocyte culture supernatant. We deduced that astrocytes not only induce the extension of cytoplasmic processes, but that profound changes in the phenotype of macrophages take place, possibly resulting in an immunologically deactivated state. Our hypothesis that the morphological transformation of macrophages and blood monocytes that was observed in our specific experimental settings is paralleled by immunological deactivation is in accordance with other findings, where the co-culture of microglial cells with astrocytes resulted in distinct functional changes that can be interpreted as “deactivation”: Recent data demonstrated that endotoxin-induced expression of nitric oxide synthase in microglial cells is inhibited by astrocyte-derived TGF-β and Aloisi et al. found that the production of IL-12 by microglial cells is inhibited in the presence of astrocytes (1, 46).

Deactivation of formerly activated microglial cells also occurs *in vivo*, and different factors with deactivating potential have been proposed to be of importance (20, 21, 35, 44): TGF-β is expressed in the lesioned CNS (21), and our own findings show that TGF-β1 is able to mimic the effects of astrocyte supernatant on adhesion molecule- and MHC-class-II-expression on rat spleen macrophages. Moreover, the partial or even complete inhibition of LPS-induced increases in MHC-class-II- or adhesion molecule expression by treatment with astrocyte culture supernatant seemed to be weakened by simultaneous incubation with neutralizing anti-TGF-β-Abs, although large inter-experiment variations made it difficult to statistically prove this preliminary conclusion. Thus, our observations suggest that TGF-β could play an important role in the process of monocyte/macrophage deactivation, and they would also be in accordance with the present opinion on the induction of ocular immune privilege, where TGF-β has been implicated as a decisive regulatory factor (41). However, it is important to note that due to differences in receptor equipment the effects of TGF-β differ in various species and cell types, e.g., human blood monocytes could react differently from rat spleen macrophages and these could again be distinct from microglia. It is also very probable that other factors act in concert with TGF-β1 to promote the deactivation of infiltrating monocytes/macrophages, and further studies utilizing neutralizing Abs or antisense strategies will shed new light on the underlying molecular mechanisms.

In summary, APCs that invade the brain parenchyma are deactivated by soluble, astrocyte-derived factors. The underlying mechanism is not restricted to the co-culture of macrophages or monocytes with astrocytes, but even plays a role in the complex microenvironment of the hippocampal slice culture with its multitude of growth factors and cytokines. Thus, the CNS cytokine network seems to be carefully orchestrated to result in strict control over endogenous and exogenous APCs. This deactivating mechanism thereby contributes to the maintenance of the brain's immune privilege.

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