Activated Mouse Astrocytes and T Cells Express Similar CD44 Variants. Role of CD44 in Astrocyte/T Cell Binding

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Abstract. The CD44 adhesion molecule is expressed by astrocytes, glial-type cells which exhibit features of accessory cells for immune responses in the central nervous system. In primary cultures of mouse astrocytes, we have observed that surface expression and mRNA levels of CD44 are induced following stimulation with either PMA, or tumor necrosis factor alpha plus gamma interferon. Comparison of CD44 splice variants expressed by astrocytes and a T cell hybridoma shows that upon activation, both cell types express a similar pattern of CD44 transcripts. Thus, in both cell types, CD44 transcripts are produced which contain additional exons, including the exon v6 (known to be expressed by in vivo activated lymphocytes and by metastatic variants of tumor cells) as well as variants of larger size. In the autoimmune disease multiple sclerosis, activated T cells cross the blood–brain barrier and lead to inflammation in the central nervous system. Analysis of mice with experimental allergic encephalomyelitis, frequently used as an animal model of multiple sclerosis, shows that CD44 is induced in vivo on glial cells surrounding inflammatory lesions. Using an in vitro model for adhesion between T cells and astrocytes, we have found a correlation between the activation state of these cells and their adhesion potential. Dose-dependent inhibition of adhesion by hyaluronate and by anti-CD44 monoclonal antibody KM81 shows that CD44 is involved in the adhesive interactions between T cells and astrocytes.

THE CD44 (Pgp-1) molecule is one of the surface antigens expressed by cells of the immune and the central nervous system (CNS)[1] (Quackenbush et al., 1985; Flanagan et al., 1989), as well as in a variety of other tissues. CD44 has been extensively studied in the lymphoid compartment, as (*a*) a molecule involved in the development and differentiation of T and B cells (Trowbridge et al., 1982; Lesley et al., 1985; Hyman et al., 1986; Miyake et al., 1990*a*); (*b*) an activation marker, increased on the cell surface following stimulation by antigen or mitogen (Budd et al., 1987; Tabi et al., 1988; Mobley and Dailey, 1992); and (*c*) an adhesion molecule involved in the interactions of lymphocytes with cells lining the so-called high endothelial venules (HEV), an important site of cell extravasation from the blood into the tissues (Berg et al., 1989), and in cell binding to extracellular matrix (ECM) molecules (collagen and fibronectin) (Carter and Wayner, 1988; Jalkanen and Jalkanen, 1992). More recently, CD44 was found to function as a receptor for hyaluronate (HA), a major component of the ECM (Lesley et al., 1990; Miyake et al., 1990*b*; Aruffo et al., 1990).

The identification of a CD44 variant (p-meta-1) capable of inducing a metastasizing phenotype when transfected into a rat carcinoma cell line (Günthert et al., 1991), as well as a number of splice variants within the extracellular membrane–proximal domain (Hofmann et al., 1991; Jackson et al., 1992; Screaton et al., 1992; Tölg et al., 1993), has brought more complexity to our understanding of the CD44 molecule. Recently, it has been shown that lymphocyte activation induces a CD44 transcript containing the v6 exon found as part of the p-meta-1 variant (Arch et al., 1992).

In the CNS, the expression of CD44 has been found to be restricted to astrocytes in the white matter (Quackenbush et al., 1985; Girgrah et al., 1991), and a number of high-grade astrocyte-type gliomas strongly express surface CD44 (Kuppner et al., 1992). Astrocytes show some features typical of accessory cells of the immune system. Firstly, they can be induced to express class II major histocompatibility complex (MHC) molecules by gamma interferon (γ-IFN) or vi-

1. *Abbreviations used in this paper*: alpha-TNF, tumor necrosis factor alpha; CHX, cycloheximide; CNS, central nervous system; EAE, experimental allergic encephalomyelitis; ECM, extracellular matrix; FCM, flow cytometry; gamma-IFN, gamma interferon; GFAP, glial fibrillary acidic protein; HA, hyaluronic acid; HEV, high endothelial venules; MHC, major histocompatibility complex.

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ral infection (Hirsch et al., 1983; Wong et al., 1984; Fierz et al., 1985; Massa et al., 1987), and tumor necrosis factor alpha (α-TNF) together with γ-IFN hyperinduces class II expression (Vidovic et al., 1990). Secondly, astrocytes are capable of presenting antigen to T cells in a MHC-restricted manner (Fierz et al., 1985). Finally, activated astrocytes can produce α-TNF, as well as a number of other cytokines (Lieberman et al., 1989). There are other interrelationships between astrocytes and the immune system. Thus, in multiple sclerosis, α-TNF is found in demyelinating plaques (Hofman et al., 1989), where astrocyte proliferation occurs (Traugott et al., 1985). Intracerebral accumulation of lymphocytes has been shown in experimental allergic encephalomyelitis (EAE) lesions (Sriram et al., 1982). Astrocytes normally participate in maintaining the integrity of the blood-brain barrier, because they interact closely, by end-foot processes, with endothelial cells, which they can induce to form tight junctions (Hertz et al., 1990). Interactions between astrocytes and lymphocytes are of importance in the context of multiple sclerosis, where such interactions may occur during the entry of activated lymphocytes into the CNS, and in the sites of lesions.

The aim of this study was to examine expression of CD44 isoforms in astrocytes, and to see whether CD44 molecules are involved in the interactions between astrocytes and lymphocytes. In primary cultures of astrocytes from the newborn mouse brain, we show that transcript levels and surface expression of total CD44 is up-regulated following activation with either the tumor-promoting agent PMA, or α-TNF plus γ-IFN. In the spinal cord of mice with EAE-induced pathology, we find that CD44 expression is strongly induced on glial cells surrounding inflammatory plaques. In addition, we show that a number of CD44 splice variants, including types containing exon v6 and of size comparable to the p-meta-1 variant, are induced in astrocytes upon activation. Rat astrocytes in culture express on the cell surface low levels of v6-containing CD44 molecule(s), recognized by the anti p-meta-1 mAB 1.1ASML, and v6 expression is increased following in vitro stimulation. Interestingly, T cells and astrocytes show a very similar pattern of CD44 expression and induction. Finally, we show by an in vitro adhesion assay that a T cell hybridoma line can adhere to an astrocyte monolayer. This interaction is HA-dependent, and can be inhibited by mAb KM81 directed to the HA-binding site of CD44. Our results therefore demonstrate that CD44 is involved in direct contacts between T cells and astrocytes.

Materials and Methods

Cell Cultures

Astrocyte cultures were established from newborn mouse brains, following a method described in the rat by Booher et al. (1972). Brain hemispheres from day 0 or 1 (C57Bl/6 × SJL) F_1 mice (bred in the LGME Facility; Strasbourg, France) were isolated, meninges removed, and a single cell suspension prepared by repeated passages through a 21G hypodermic needle. Total cells were plated on poly-L-lysine (Sigma Immunochemicals, St. Louis, MO) coated 100 × 20-mm plastic dishes (Falcon, Becton-Dickinson, NJ) or chamber slides (Polylabo, Strasbourg, France) in Waymouth medium (Gibco, Life Technologies Ltd., Paisley, Scotland) supplemented with 10% FCS, at a density corresponding to one brain to 18 ml medium (∼10^6 cells/ml), and incubated at 37°C in a 5% CO_2 humidified atmosphere. In these culture conditions, even after 3-4 d, astrocytes were the predominant cell type. Thus, >95% of cells stained with an antibody to glial fibrillary acidic protein (GFAP). Very few macrophages were present, as assessed by staining for MAC-1, and microscopic examination showed that few (<1%) fibroblasts were present.

The H11.1 T cell hybridoma, generated by fusing the AKR thymoma BW5147 with fetal mouse thymocytes grown in IL-2 as described (Ceredig et al., 1989), was grown in supplemented DME (Cerottini et al., 1974) containing 10% FCS. Cells were stimulated using the tumor-promoting agent PMA (Sigma Immunochemicals) at a concentration of 50 ng/ml for astrocyte cultures, and 10 ng/ml for the H11.1 cell line. Recombinant human α-TNF and mouse γ-IFN (gifts from Dr. C. Benoist, LGME) were added at concentrations of 10 ng/ml and 200 IU/ml, respectively. The protein synthesis inhibitor cycloheximide (CHX) was used at 10 ng/ml.

Immunostaining and FCM Analysis

Astrocyte cultures grown on poly-L-lysine-coated chamber slides were stained for CD44 surface expression using the rat anti-mouse mAb IM7 (a gift of Dr. R. Hyman), followed by FITC-conjugated sheep anti-rat Ig (Silenus, Melbourne, Australia). The anti-GFAP antibody was rabbit Ig (Dako, Biosis, France). Cells were stained for GFAP after permeabilization in 0.3% saponin, followed by Texas red-conjugated goat anti-rabbit $F(ab')_2$ (Jackson ImmunoResearch Labs, Inc., West Grove, PA). Cells were fixed with 1% paraformaldehyde in PBS, and photographs were taken under a fluorescence microscope (Zeiss, Strasbourg, France).

Spinal cords and brains from normal and EAE mice were provided by Dr. H. Bodmer, 10-μm frozen sections were fixed with 1% paraformaldehyde and stained for CD44 expression. Non-specific fixation of biotin-labeled IM7 was prevented by first incubating sections in 1% BSA. Fixed antibody was revealed by avidin-phosphatase (Vectastain ABC; Vector Laboratories, Burlingame, CA), after blocking of endogenous phosphatase activity with 50 mM levamisole (Sigma Immunochemicals). Sections were lightly counterstained with hematoxylin.

For flow cytometry (FCM) analysis, astrocytes were trypsinized (3 min, 0.04% trypsin in PBS) from dishes, dissociated, and washed in medium, and ∼5 × 10^5 cells were stained by indirect immunofluorescence. Single staining for CD44 and GFAP was done using the reagents described above for Fig. 2, and with biotin-labeled IM7 followed by Av^{FITC} (Becton-Dickinson & Co., Mountain View, CA) for Fig. 1. The mouse antibody 1.1ASML directed to the v6-encoded region of CD44 was described by Matzku et al. (1986). Rat astrocytes were double stained with biotin-labeled 1.1ASML and Av^{FITC}, and with anti-GFAP antibody as described above. Staining for the MAC-1 Ag was with the rat anti-mouse MAC-1 mAb MI-70 (a gift from Dr. I. F. C. McKenzie), followed by FITC-conjugated sheep anti-rat IgG. FCM analysis was carried out on an ODAM ATC 3,000 flow cytometer (Wissembourg, France).

RNA Preparation and Northern Blot Analysis

RNA was purified by centrifugation on a 5.7 M cesium chloride cushion as described (Maniatis et al., 1982), and separated according to size on a 1% agarose formaldehyde gel which was blotted onto a Hybond-N nylon filter (Amersham, Les Ulis, France). Probes for hybridization were obtained by random priming of the CD44 (Pgp-1) cDNA 1.3-kb EcoRI fragment from plasmid MHR6-Prk5 (Zhou et al., 1989), the variant CD44 region exons v4 to v10 cDNA, and the β-actin cDNA (gift from Dr. A. Hanauer, LGME). Bands on X-OMAT Kodak films (Rochester, NY) exposed for varying lengths of time were scanned using a Biocom Compaq gel scanner, and the band intensities analyzed using the program ELPHOR (Biocom, Les Ulis, France).

PCR Amplification of CD44 Variant Region

Oligonucleotides were used hybridizing to CD44 constant region 5' and 3' of the variant region, and in exons v6 and v7 of the variant region (see Fig. 7 *a*). Aliquots of total RNA samples (∼1 μg) were reverse transcribed using 20 μg/ml oligodT primer in 50 mM Tris HCl, 20 mM KCl, 10 mM $MgCl_2$, 5 mM DTT, and 1 mM of each dNTP. AMV reverse transcriptase (9 U) and ribonuclease inhibitor (6 U) (Amersham) were added in 20 μl final volume, and incubation was for 40 min at 42°C. The reaction was stopped by adding 80 μl H_2O, and 1 μl of this cDNA preparation was submitted to PCR amplification. The PCR amplification was carried out in a buffer containing 10 mM Tris HCl, 50 mM KCl, 2 mM $MgCl_2$, 1% gelatin and 200 μM of each dNTP, 1 μM of each primer pair and 0.5 U of Taq polymerase (Perkin Elmer Cetus, Norwalk, CT) added in a final volume of 20 μl. Samples were denatured 10 min at 92°C, followed by 35 cycles of 30" at 92°C, 20" at 60°C, and 1 min at 72°C, with a 2" extension at each cycle, and

a 10-min elongation at 72°C. PCR products were then separated on a 2% agarose gel, blotted onto Hybond-N+ (Amersham), and hybridized to CD44 variant region cDNA probes corresponding to exons v6-7 and v10 obtained by random-priming. Filters were exposed to Kodak X-Omat films for intervals of several hours to 2 d.

Adhesion Assays

Astrocytes were trypsinized from primary cultures, seeded in flat-bottomed 96-wells plates (Falcon, Becton-Dickinson, NJ), and allowed to grow for 3 d, to obtain a confluent cell layer. The adhesion protocol was a modification of that described by Lepesant et al. (1990). Plates were kept on ice to minimize metabolic activity of the cells. Medium was aspirated and replaced by graded numbers of ^{51}Cr (Amersham)-labeled H11.1 T cells in 100 μl cold culture medium. Cells were allowed to settle for 1 h, and then wells were filled with sufficient cold PBS to obtain a positive meniscus, and carefully sealed with parafilm, avoiding the formation of air bubbles. The plates were then inverted and centrifuged for 5 min at 500 rpm, and flicked to eliminate all medium and unbound cells. The remaining bound cells were lysed in 10% NP-40, and the radioactivity was counted. The percentage of adhesion was calculated as a ratio between the cpm from bound cells (triplicate or quadruplicate samples) and the total input cpm. Negative controls included H11.1 cells treated with 0.2% trypsin for 30 min at 37°C, and also H11.1 cells added to empty plastic wells. When needed, PMA was added to astrocytes or H11.1 cells ~15 h before the assay. Hyaluronidase treatment was for 1 h at 37°C, in medium containing 1 μg/ml hyaluronidase (provided by M. Gilbert, LGME). HA (see Fig. 8 *c* for concentrations) (Sigma Immunochemicals) was added in the wells together with the H11.1 cells just before the assay. mAb inhibition assays were performed in the presence of culture supernatants of anti-CD44 mAbs KM81 or KM201 (kind gifts of Dr. P. Kincade), or IM7. Preincubation of either the astrocyte monolayer, or the H11.1 T cells in the presence of culture supernatants of monoclonal antibodies KM81 or IM7 was for 1 h on ice. The remaining free antibodies were washed away with culture medium before the addition of T cells into the microwells.

Results

CD44 Is Expressed by Primary Cultures of Mouse Astrocytes

Differentiated astrocyte populations expressing GFAP were obtained within 2–3 d of culture in 10% serum (Lillien and Raff, 1990). Fig. 1 (*left*) shows a typical display of forward versus side scatter signals from in vitro cultured astrocytes. Based on the cytogram display, cells could be subdivided into three subpopulations of small (No. 1), medium (No. 2), and large (No. 3) cells. Such subpopulations could correspond to those defined by morphological criteria, namely fibrous- and protoplasmic-type astrocytes. Larger cell populations stained more brightly for GFAP, as mean fluorescence intensities of populations No. 1, No. 2, and No. 3 were 102, 139, and 205, respectively (*middle*). Expression of CD44 increased with increasing cell size, thus 59% of population No. 1, 75% of No. 2, and 92% of No. 3 were CD44+ (right). During development in culture, FCM analysis showed that CD44 expression appeared simultaneously with GFAP (not shown).

Induction of CD44 Expression by Astrocytes

CD44 expression on the surface of T lymphocytes has been shown to increase following stimulation with antigen, or PMA and calcium ionophore (Budd et al., 1987; Tabi et al., 1988; Mobley and Dailey, 1992). PMA treatment of astrocytes activates protein kinase C and induces cell proliferation (Murphy et al., 1987). To see whether activated astrocytes show an increase in CD44 expression, primary cultures were treated with PMA and analyzed for surface CD44 expression. As shown in Fig. 2, CD44 was weakly expressed by total unstimulated astrocytes. It should be noted that indirect staining with IM7 SN (as shown in Fig. 2) was always weaker than by the biotin-avidin method. At 4 h after PMA stimulation, the staining intensity had increased and 69% of cells were positively stained. The increase in CD44 expression was seen in three independent experiments.

To look at the distribution of CD44 on cultured astrocytes, fluorescence microscopy was carried out. By this technique, CD44 was found to be unequally distributed on the surface of the cells, being more highly expressed on astrocyte processes and at sites of contact between adjacent cells (Fig. 3).

It is known that γ-IFN together with α-TNF superinduces MHC class II expression on astrocytes (Vidovic et al., 1990). In addition, α-TNF can be produced by activated astrocytes, and is detected in multiple sclerosis lesions (Lieberman et al., 1989; Hofman et al., 1989). To see what effect

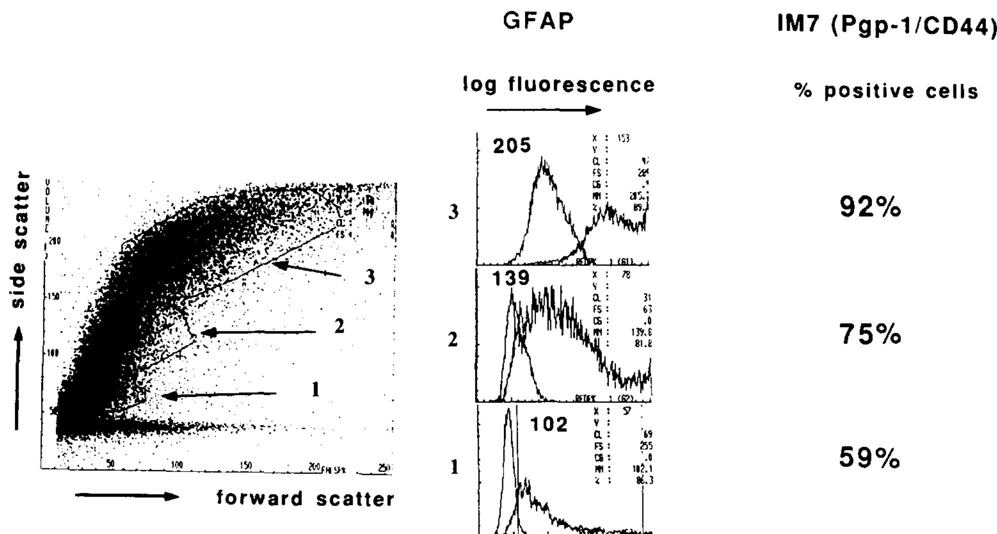

Figure 1. Phenotype of astrocytes in primary cultures from newborn mouse brain cells. Cells from a 2-wk-old primary culture were stained in suspension for the astrocytic marker GFAP and for CD44, and analyzed by FACS. Due to heterogeneity in size and granulation, represented by forward and side scatter, the bulk was divided into three populations by gating; shown are the fluorescence histograms for GFAP (mean fluorescence intensities are indicated) and the percentage of CD44+ cells (staining with the biotinylated IM7 mAb) in each subpopulation.

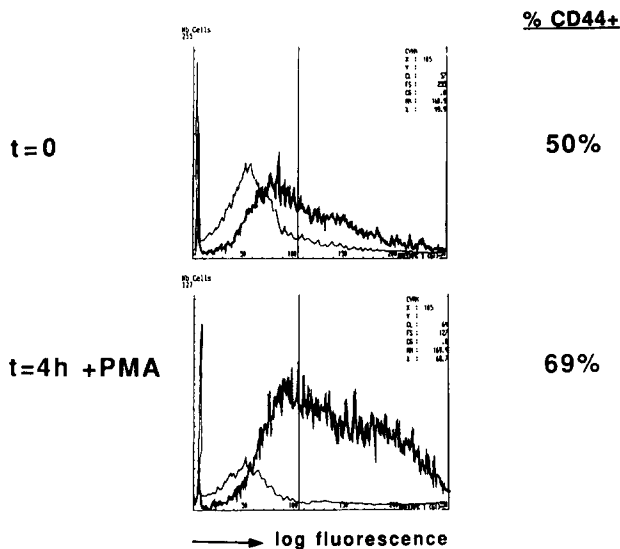

Figure 2. PMA stimulation of astrocytes primary cultures induces CD44 surface expression. After 4 h of PMA treatment, cells from 1-m-old primary cultures were stained in suspension using the IM7 mAb in culture supernatant, and analyzed by FACS for CD44 surface expression. The percentage of positive cells is indicated.

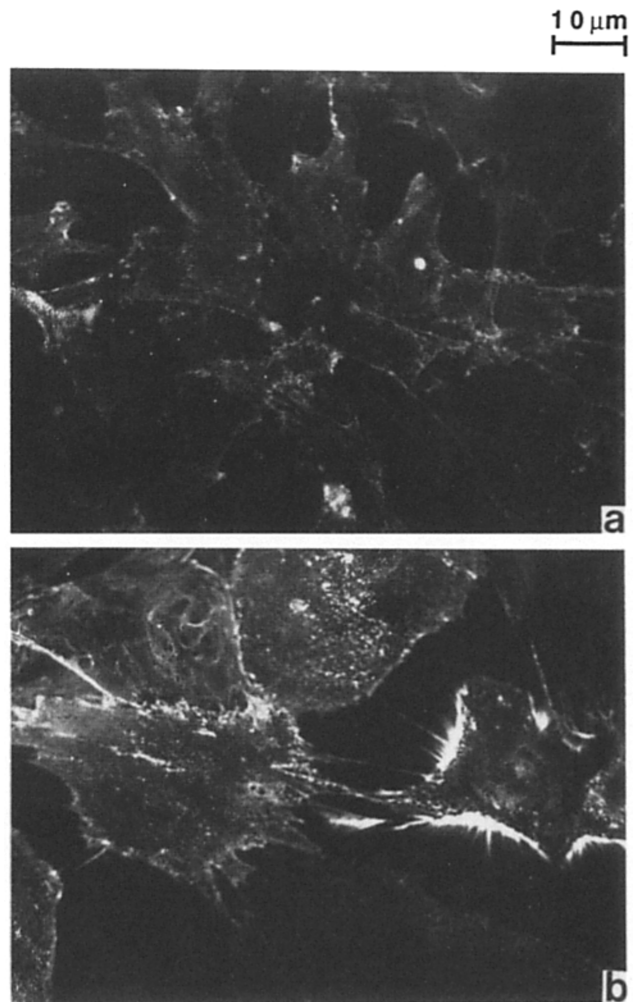

Figure 3. CD44 surface expression in adherent astrocytes primary cultures, either unstimulated (*a*), or stimulated overnight with r-Hu-α-TNF + Mu-γ-IFN (*b*). Fluorescence immunostaining using biotin-labeled IM7 mAb.

these cytokines had on CD44 expression by astrocytes, r-Hu-α-TNF, Mu-γ-IFN, or both factors were added to astrocyte primary cultures. As shown in Fig. 3 *b*, this treatment resulted in the induction of CD44 surface expression, which was again more brightly expressed along astrocyte processes and sites of intercellular contact.

To see whether CD44 is induced on astrocytes in vivo, sections of spinal cord from either normal (Fig. 4, *a* and *c*) or mice with EAE-induced pathology (Fig. 4, *b* and *d*) were subjected to immunophosphatase staining. As shown in Fig. 4, *b* and *d*, CD44 was strongly expressed by glial cells surrounding inflammatory lesions. The level of CD44 expression on these glial cells was clearly above that found in the white matter of normal mice (Fig. 4, *a* and *c*).

Northern Blot Analysis of CD44 Transcripts in Activated Astrocytes

We were interested to see whether in astrocytes, as in T cells (Haegel and Ceredig, 1991), cell activation resulted in an increase in CD44 transcripts. Therefore, Northern analysis was carried out of total RNA from unstimulated and PMA-stimulated primary astrocyte cultures. In T cells, we have already shown that the predominant CD44 transcripts are of 4.5, 3.5, and 1.6 kb with the 4.5-kb transcript being the dominant form. In contrast, in fresh unstimulated astrocytes, the only clearly distinguishable CD44 transcript was of 4.5 kb (Fig. 5 *a*); transcripts of lower sizes were present but less distinguishable than in T cells. Activation by PMA resulted in a marked increase of CD44 transcripts. In this experiment (Fig. 5), the astrocytes used had been in culture for 14 d before PMA stimulation. The ratio of CD44/actin transcripts, as determined by scanning autoradiograms, was 6.2-fold after 4-h stimulation and 18.5-fold after 18 h. In agreement with our previous results with T cells (Haegel and Ceredig, 1991), this increase in CD44 transcripts was prevented by the addition of the protein synthesis inhibitor CHX. Thus compared with control cultures, in the presence of CHX, CD44 transcripts levels had increased only 2.5-fold after 4 h and 2.6-fold after 18 h, respectively. This result suggests that de novo protein synthesis was required for the increase of CD44 transcription after stimulation.

In addition to hybridization with an actin probe (not shown), the same filters were hybridized with a Thy-1 probe. Thy-1 is a cell surface antigen expressed by mouse and human astrocytes. As shown in Fig. 5 *b*, stimulation with PMA in the presence or absence of CHX did not significantly alter Thy-1 transcript levels.

In the experiment shown in Fig. 5, the astrocytes used were from 2-wk-old cultures. At this time it is known that astrocytes are optimally responsive to PMA (Honegger et al., 1986). Later experiments used astrocytes from 3–4-wk-old cultures and the increase in CD44 transcripts was relatively more modest. Thus astrocytes were stimulated with either PMA or γ-IFN and α-TNF alone or in combination. With γ-IFN or α-TNF alone, the increase in CD44 transcripts,

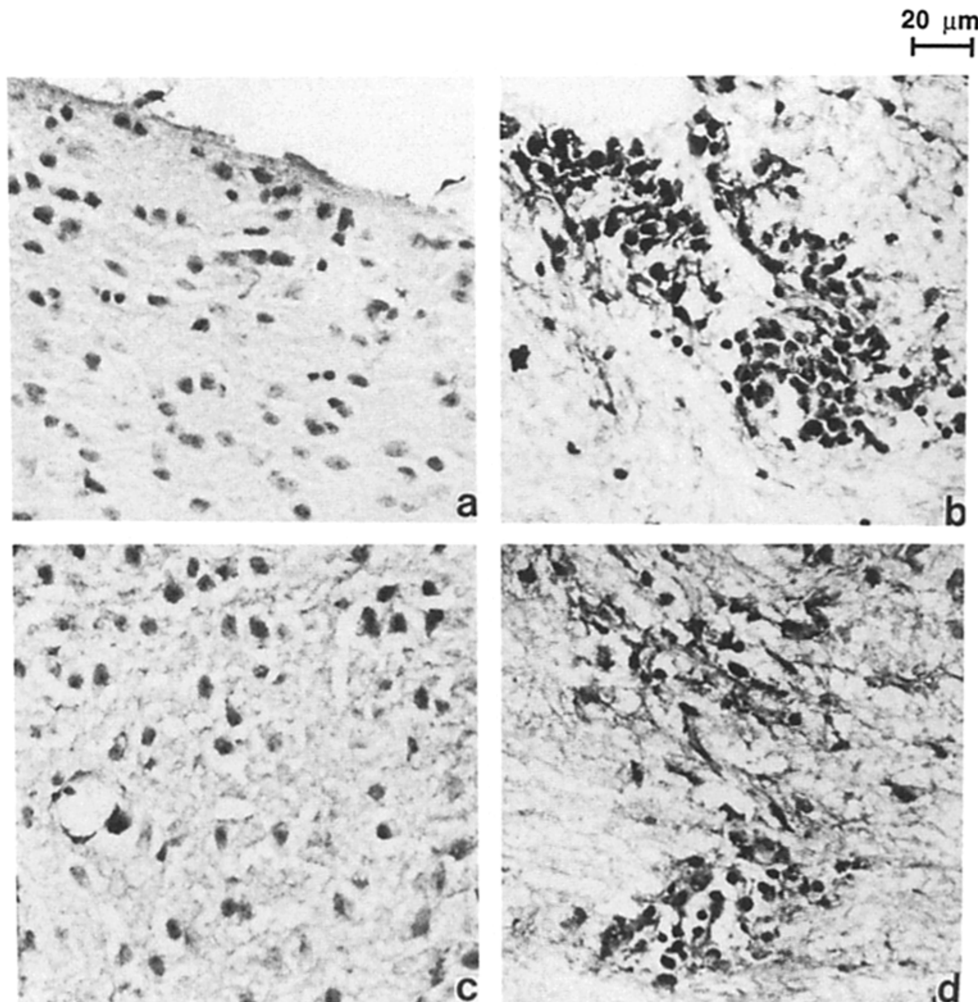

Figure 4. CD44 expression is induced in EAE lesions. Immunophosphatase staining for CD44 in frozen sections of normal (*a* and *c*) and EAE mouse (*b* and *d*) spinal cord, counterstained with hematoxylin. In inflammatory lesions, where mononucleated cells localize, CD44 is strongly expressed by the surrounding glial cells.

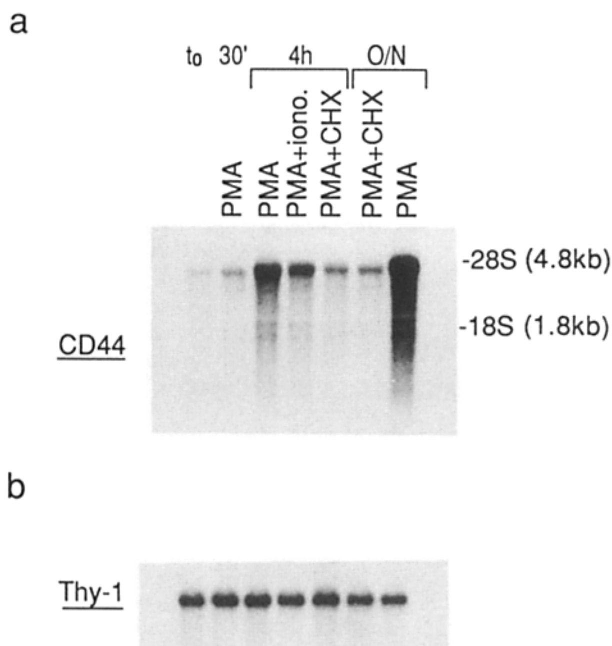

Figure 5. Northern Blot analysis of CD44 and Thy-1 transcripts following PMA stimulation of astrocytes primary cultures. Cells in 2-wk-old primary cultures were stimulated with PMA in the presence or absence of the protein synthesis inhibitor CHX or calcium ionophore, and total RNA was isolated at the times indicated. The filter was hybridized using cDNA probes for CD44 (*a*) and Thy-1 (*b*). Hybridization using a β-actin probe (not shown) indicated that equal amounts of RNA were loaded.

compared to actin control, was 1.3- and 2.2-fold, respectively. The combination of γ-IFN and α-TNF had an additive effect on CD44 transcripts levels, resulting in a 2.7-fold induction. Fig. 6 shows the results from a minimum of three experiments, in which the CD44/actin ratio was determined in astrocytes stimulated overnight with the indicated agents. Thus PMA consistently increased CD44 transcripts, and α-TNF plus γ-IFN always resulted in an additive effect.

Variant CD44 Molecules in Activated Astrocytes

Because of the increasing variety of known CD44 splice variants (Günthert et al., 1991; Hofmann et al., 1991; Jackson et al., 1992; Arch et al., 1992; Screaton et al., 1992; Tölg et al., 1993), we decided to determine which CD44 variants were being expressed by unstimulated and stimulated astrocytes. Importantly, we asked whether astrocytes and T cells may be expressing the same variant forms of CD44. Initially, Northern blots of total RNA were performed using a probe for the v4 to v10 exons. The signals obtained were too weak

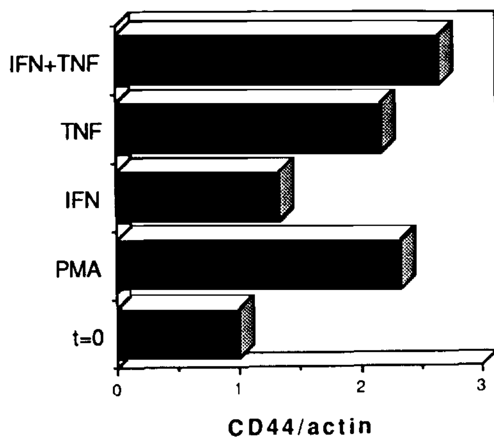

Figure 6. Induction of CD44 transcripts in stimulated astrocyte primary cultures. Northern Blots from 3-wk-old astrocyte primary cultures stimulated overnight with PMA or Mu-γ-IFN, r-Hu-α-TNF, and both factors, were scanned and the intensities of CD44 bands were compared to those of β-actin. Results shown are the mean of three experiments.

for the interpretation of results, although "standard-type" CD44 could be easily detected on the same filters, suggesting that mRNAs encoding splice variants of CD44 are present in lower amount than standard CD44 form within the cells. Therefore, we undertook a PCR strategy. As shown in Fig. 7 *a*, combinations of four oligonucleotides (*A–D*) were used in these PCR experiments. Thus oligos A and D (Fig. 7 *b*, lanes *3* and *6*) amplified from the 5′ constant through to the 3′ constant region of CD44 and should theoretically amplify all variable domain. Oligos B and D (Fig. 7 *b*, lanes *1* and *4*) would amplify from v6 to the 3′ constant region, whereas A and C (in Fig. 7 *b*, lanes *2* and *5*) amplify from the 5′ constant region up to and including v7. These PCR amplified fragments were Southern blotted with probes to either v6-7 (Fig. 7 *b*, *top*) or v10 (Fig. 7 *b*, *bottom*).

As shown in Fig. 7 *b* (lanes *1* and *4*), two bands of 320 and 190 bp hybridizing to probes v6-7 (Fig. 7 *b*, *top*) but not v10 (Fig. 7 *b*, *bottom*) were observed in PMA-stimulated astrocytes. Non-stimulated astrocytes did not usually express these "meta-type" CD44 variants, of sizes corresponding to the p-meta-1 (Günthert et al., 1991) and to the v6-containing isoform shown to be expressed by in vivo activated lymphocytes (Arch et al., 1992). In contrast, the H11.1 T cell line, either unstimulated or stimulated, consistently expressed these CD44 variant forms. The band of ∼800 bp (Fig. 7 *b*, lanes 4, *top* and *bottom*) containing v6 (*top*), v10 (*bottom*) plus additional exons is induced in both astrocytes and H11.1 cells. Lanes *2* and *5* (*top*) show that variants containing v7 were more abundant in stimulated vs. unstimulated astrocytes, but remained the same in H11.1 cells. A band around 500 bp, a size corresponding to the p-meta-1 variant, is clearly induced in activated astrocytes. A smaller band of 280 bp indicates the presence of a variant containing one additional exon 5′ to the v7 exon, expressed by astrocytes and H11.1 cells regardless of their activation state. In lanes *3* and

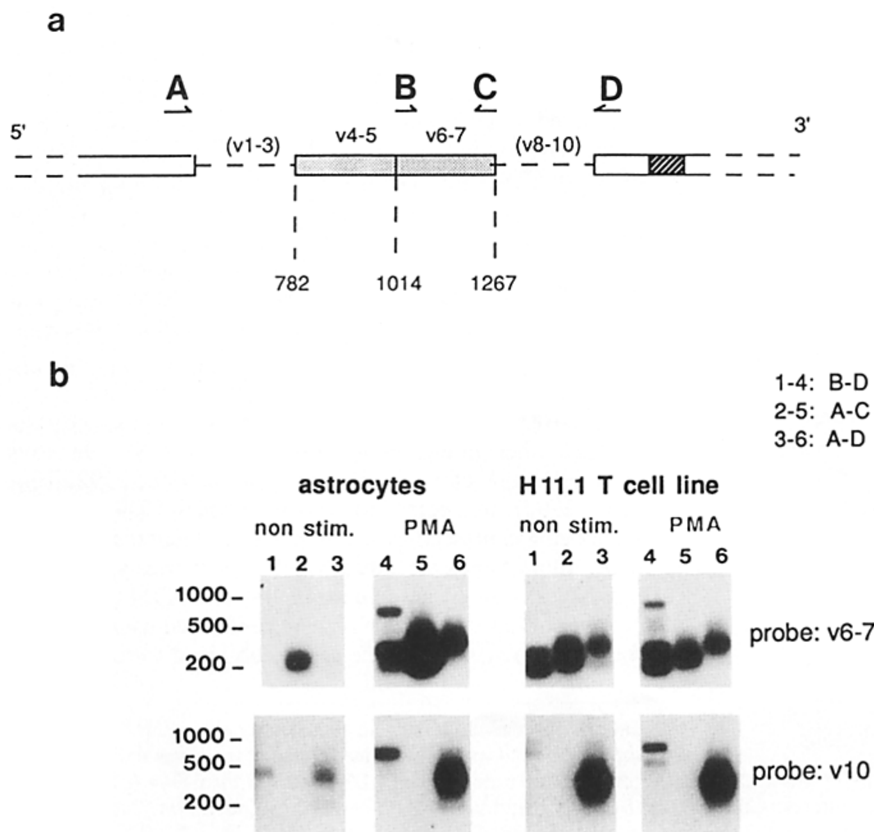

Figure 7. PCR detection of CD44 variants expressed in astrocyte primary cultures and in the H11.1 T cell line stimulated with PMA. (*a*) PCR strategy used to distinguish between "meta-types" and larger CD44 variants. A schematic structure of the p-meta-1 variant (Günthert et al., 1991) is represented; "standard" CD44 sequence (*open bars*), exons from the variant region (*dark bars*), and the transmembrane region (*hatched bar*) are shown. Localization of the putative additional exons (*hatched lines*) identified in larger CD44 variants is according to Tölg et al., 1993. Oligonucleotides A, B, C, and D hybridize to positions 653–680, 1039–1070, 1146–1173, and 1328–1356, respectively. (*b*) cDNA from non-stimulated or PMA activated astrocytes and T cell line H11.1 were amplified using the primer pairs indicated. Amplification using oligos B and D (*1–4*) permits to distinguish between "meta-type" variants, producing bands of 320 bp (p-meta-1) and 260 bp (v6-containing variant) (Arch et al., 1992) hybridizing to the v6-7 probe but not to the v10 probe, and other variants producing larger bands hybridizing to both probes. Amplification using oligos A and C (2–5) identifies variants containing exon v7. Use of the two external oligos A and D (*3–6*) permits amplification of the whole CD44 variant region.

6, only one main band of around 400 bp was detected. We failed to amplify larger variant forms with these oligos. Nevertheless, lanes 3 and 6 (*top*) show that a variant containing exons v6 and/or v7, and of size corresponding to the v6-containing variant (Arch et al., 1992), is induced upon astrocyte activation. This form is constitutively expressed by H11.1 cells. In addition, a v10-containing variant, weakly expressed by astrocytes, is induced upon PMA stimulation (*bottom*). Although not quantitative, the PCR results shown in Fig. 7 *b* were routinely obtained. As mentioned above, some non-stimulated astrocytes expressed weakly v6-7-containing transcripts.

To detect variant forms of CD44 on the cell surface of astrocytes, specific antibodies are required which are not yet available for the mouse molecules. The mAb 1.1ASML (Matzku et al., 1989) recognizes a region encoded by the variant region exon v6, present in the p-meta-1 variant of CD44. The 1.1ASML epitope is induced in activated rat lymphocytes following antigenic stimulation in vivo (Arch et al., 1992). Therefore, to demonstrate the surface expression of v6-containing CD44 molecules, rat astrocytes were stained by two color immunofluorescence for GFAP and 1.1ASML. Fig. 8 *a* shows the GFAP staining profile, and Fig. 8 *b* the 1.1ASML profile of GFAP-positive cells. Unstimulated astrocytes stained very weakly with 1.1ASML, but the presence of v6-containing CD44 was clearly detectable on the surface of PMA-activated astrocytes. These results demonstrate the expression of v6-containing CD44 variant on the surface of stimulated rat astrocytes.

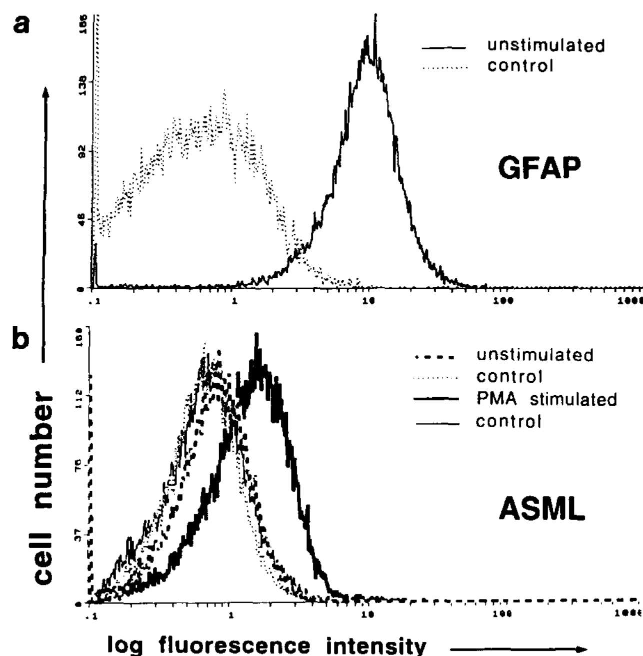

Figure 8. Induction of v6-containing CD44 on the surface of stimulated rat astrocytes. Rat astrocytes in 2-wk-old primary cultures were stained in two colors for the astrocytic marker GFAP (*a*) and for the v6-encoded region of CD44 recognized by mAb 1.1ASML. The 1.1ASML staining profile of the GFAP-positive cells is shown in (*b*), before (*dotted*) and after (bold) 24 h of in vitro stimulation with 50 ng/ml PMA.

In conclusion, astrocytes can express several variant forms of CD44, including "meta-type" variants, and the latter are increased in PMA-stimulated cells. The 800-bp PCR fragment (Fig. 7 *b*, lanes *4*) containing exons v6 to v10 was also found to be induced in astrocytes by α-TNF and γ-IFN (not shown). In general, there is a striking similarity between the profile of CD44 variants expressed by activated astrocytes and activated T cells.

Involvement of CD44 in Adhesion between Astrocytes and a T Cell Line

In the context of inflammatory diseases of the CNS, most notably EAE, the direct interaction between activated T cells and astrocytes may play an important role in the localization of T cells to sites of brain injury. α-TNF expression and astrocyte proliferation have been detected in areas surrounding the demyelinating plaques in the CNS (Hofman et al., 1989; Traugott et al., 1985).

To study the interaction between T cells and astrocytes in more detail, an in vitro adhesion assay was developed. In this assay, ^{51}Cr-labeled H11.1 T hybridoma cells were allowed to adhere to monolayers of unstimulated or stimulated astrocytes. Using this assay, we could show that the adhesion of H11.1 cells to astrocytes was augmented following stimulation of either H11.1 cells or astrocytes (Fig. 9 *a*). Stimulation of both H11.1 and astrocytes resulted in increased adhesion. Control experiments showed that spontaneous ^{51}Cr release by H11.1 cells did not increase following PMA stimulation (not shown). To show the role of CD44 in this adhesion process, H11.1 cells were allowed to adhere to PMA-stimulated astrocytes which had been either left without further treatment, or after treatment with hyaluronidase (Fig. 9 *b*). Treatment with hyaluronidase clearly inhibited the adhesion of H11.1 cells to PMA-activated astrocytes. Addition of the CD44 ligand HA led to a dose-dependent decrease in cell adhesion (Fig. 9 *c*). Control experiments showed no increased astrocyte detachment following either PMA treatment or addition of HA. After PMA treatment (Fig. 9 *c*, *right*), adhesion was more resistant to blocking by HA, suggesting that other adhesion molecules or non-HA binding CD44 variants may participate in the binding. Finally, anti-CD44 antibodies were tested for their ability to inhibit the binding between astrocytes and H11.1 cells. Presence of the KM81 mAb inhibited in a dose-dependent fashion the adhesion of H11.1 cells to astrocytes (Fig. 9 *d*, *left*). This anti-CD44 antibody is known to recognize the HA-binding domain of CD44 and importantly stains both H11.1 cells (Fig. 9 *d*, *right*) as well as astrocytes (not shown). In contrast, the KM201 mAb did not inhibit adhesion and did not stain brightly neither H11.1 cells (Fig. 9 *d*, *right*) nor astrocytes (not shown). The IM7 mAb did not inhibit adhesion (not shown), but did stain both astrocytes (Figs. 1 and 2) and H11.1 cells (Haegel and Ceredig, 1991). Pretreatment of either the astrocyte monolayer, or the H11.1 T cells with mAbs KM81 or IM7 (Fig. 9 *e*) showed that CD44 molecules on both cell types participated in the adhesion.

Discussion

We have examined CD44 expression by astrocytes in primary cultures of newborn mouse brains. In 10% FCS

medium, mature astrocytes expressing GFAP are obtained within 3–4 d, the differentiation process being much more rapid than in vivo (Lillien and Raff, 1990). The resulting cell population is heterogenous in size and CD44 expression, and the highest percentage of CD44$^+$ cells is found among the large cell population (Fig. 1). This may reflect a different activation state among distinct populations, larger cells being a more "active" population, a situation analogous to cultures of lymphocytes. A number of astrocyte subtypes have been described, namely fibrous- and protoplasmic-types, which differ by their morphology (Tardy, 1991). Our results suggest that these subtypes show a difference in surface expression of CD44.

We have found that astrocytes stimulated with PMA or α-TNF and γ-IFN increase not only surface expression (Figs. 2 and 3) but also CD44 transcript levels (Figs. 5 *a* and 6). This phenomenon is more striking in 2 wk-old primary cultures than in older cultures. This difference could be because cells in 2-wk-old cultures are more actively dividing and more sensitive to PMA stimulation (Honegger, 1986).

There is an interesting similarity in the pattern of CD44 transcripts expressed in astrocytes and in T cells (Haegel and Ceredig, 1991). In T cells a 4.5-kb mRNA is predominantly produced, together with a number of smaller size transcripts. These smaller CD44 transcripts could correspond either to varying degrees and sites of polyadenylation (Schtivelman and Bishop, 1991) and/or to different splice variants of the CD44 molecule. This analogy is strengthened by the finding that, in astrocytes as in T cells, CD44 expression is upregulated by PMA stimulation. In both cell types,

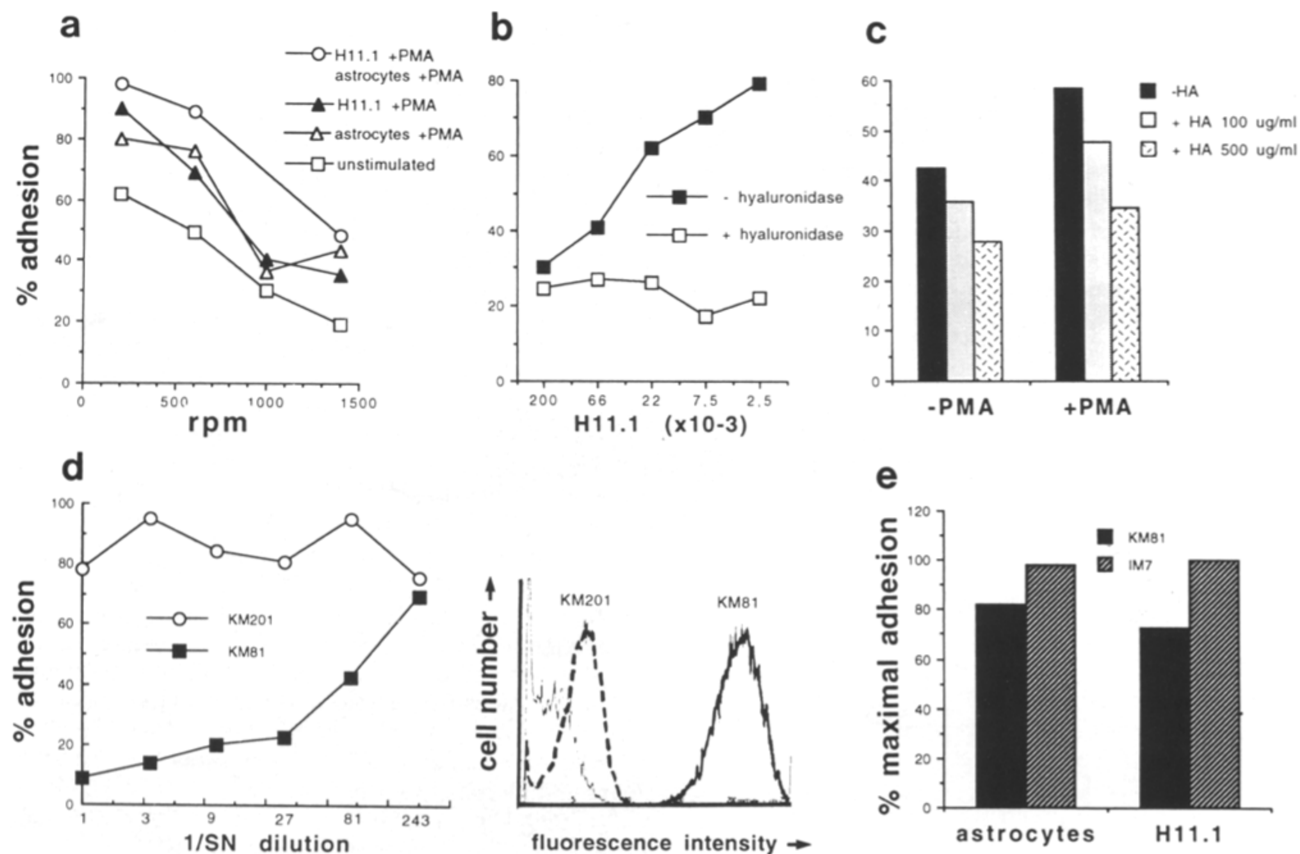

Figure 9. Adherence of the T cell line H11.1 to astrocyte monolayers. (*a*) Adhesive interaction is increased after PMA activation. 10^4 H11.1 cells were added per microwell containing monolayers of astrocytes from primary cultures. Dissociation curves (Lepesant et al., 1990) were obtained by spinning reversed plaques for 5 min at 200, 600, 1,000, and 1,400 rpm, and the percentages of remaining bound H11.1 cells were calculated. Open circles correspond to unstimulated astrocytes and T cells; astrocytes (△), H11.1 T cells (▲), and both cell types (□) were PMA-stimulated 15 h before the experiment. (*b*) Adhesion is inhibited by hyaluronidase treatment of the astrocyte monolayer. Graded numbers of H11.1 T cells were added on PMA-activated astrocyte monolayers, with (□) or without (■) previous hyaluronidase treatment of the astrocytes, and the percentages of remaining bound H11.1 cells were determined. (*c*) Adhesion is inhibited in the presence of HA. 10^4 H11.1 T cells were added per microwell containing astrocyte monolayers, either PMA activated (*right*) or unstimulated (*left*), and incubated in the presence of HA at 100 μg/ml (*grey bars*), 500 μg/ml (*hatched bars*) or in the absence of HA (*black bars*). The percentages of bound cells were determined as previously. (*d*) Inhibition of adhesion in the presence of mAb KM81. Serial dilutions of KM81 (■) or KM201 (○) culture supernatants were added to the microwells in the adhesion assay (*left*). FCM analysis (*right*) showed that KM81 mAb brightly stained H11.1 cells (*bold profile*), whereas KM201 did not (*dotted profile*). (*e*) Pretreatment of either the astrocytes, or the H11.1 T cells with mAb KM81 inhibits adhesion. The astrocyte monolayer, or the H11.1 cells, were preincubated for 1 h on ice in the presence of mAb supernatants KM81 or IM7. Cells were washed before addition of 10^5 H11.1 cells into the microwells. The percentages of maximal binding which was obtained in the absence of mAb pretreatment were calculated from two independent experiments.

this induction is inhibited by CHX, showing that it is de novo protein synthesis dependent. However, in contrast to our results with T cells (Haegel and Ceredig, 1991), PMA stimulation of astrocytes did not decrease Thy-1 transcript levels (Fig. 5 *b*), reflecting the complex regulation of the Thy-1 gene in different mouse tissues. It is known that Thy-1 expression in the brain, thymus, and peripheral T cells is regulated by tissue-specific enhancers present in distinct introns of the Thy-1 gene (Vidal et al., 1990).

A PCR strategy was used to determine which CD44 splice variants were transcribed in astrocytes and in H11.1 T cell hybridoma cells, before and after in vitro cell activation. The splice variants expressed by both cell types upon activation were similar (Fig. 7 *b*). Interestingly, "meta-type" variants containing exons v6 and/or v7 but not v10 can be expressed by unstimulated cells, and they appear to be induced in astrocytes activated with PMA or with α-TNF and γ-IFN. That v6-containing CD44 appears on the surface of activated astrocytes was confirmed by staining rat astrocytes with the mAb 1.1ASML (Fig. 8 *b*), which is known to detect p-meta-1 and the v6-containing variant of rat CD44. In view of the recent study (Arch et al., 1992) showing that a CD44 variant containing exon v6 is induced in the lymphocyte compartment following antigenic stimulation, it is likely that this feature can be extended to astrocytes in the neuroglial compartment. Moreover, we found that larger CD44 variants, containing exons v6 and v10, were also induced upon activation of both astrocytes and the H11.1 T cell line. Attention must be pointed to possible contamination of astrocyte primary cultures with different cell types. However, similar amounts of CD44 variant forms could be amplified using equal quantities of RNA from activated H11.1 cells and activated astrocytes. Therefore, possible contamination of the primary astrocytes cultures by macrophages, which never exceeded 3%, and fibroblast ($<$1%), is unlikely to explain the results obtained in our PCR experiments. In addition, two color FCM (Fig. 8) directly demonstrates that upon stimulation, astrocytes defined as GFAP-positive cells express a v6-containing, meta-type CD44 variant. Therefore, we believe that our PCR experiments faithfully reflect the expression of CD44 variants in astrocytes.

Our studies demonstrate that α-TNF and γ-IFN have an inducing effect on CD44 expression. As mentioned previously these cytokines have been shown to play a major role in cell activation, inflammation and immune reactions within the CNS. We show that they can induce an increase in CD44 surface expression as well as transcript levels (Figs. 3 and 6). When both α-TNF and γ-IFN are present, the induction is quantitatively stronger than that observed with PMA, and these cytokines appear to have an additive effect on CD44 mRNA induction. α-TNF and γ-IFN have been shown to work on cell activation via numerous mechanisms, including effect on Na^+/H^+ transport and inhibition of lipoprotein lipase (Chung et al., 1991; Beutler et al., 1985). A detailed study of the CD44 promoter is necessary to determine whether particular consensus sequences are responsible for CD44 induction by α-TNF and γ-IFN, directly or indirectly. Interestingly, astrocytes from EAE-susceptible and -resistant strains show differential expression of α-TNF (Chung et al., 1991), and an antibody to α-TNF has been shown to prevent transfer of EAE (Ruddle et al., 1990). Our results suggest that in vivo, expression of the CD44 adhesion molecule by astrocytes is increased in conditions where α-TNF and γ-IFN are present, as in inflammation, gliosis, and immune reactions in the CNS.

Immunofluorescence microscopy shows that expression of CD44 is increased at sites of contact between cells and along astrocyte processes, suggesting a role for CD44 in homotypic adhesion. Immunoperoxidase staining of normal mouse spinal cord sections (Fig. 4, *a* and *c*) indicated that CD44 was expressed in the white matter and in perivascular areas. Interestingly, we show that CD44 expression is strongly induced on glial cells surrounding inflammatory lesions in the white matter of EAE mouse spinal cord (Fig. 4, *b* and *d*). As mentioned above, astrocytes have been reported to interact closely with endothelial cell types in the brain through foot processes, and to induce the formation of tight-junctions (Hertz et al., 1990). Thus, a central role in the control of exchanges between the immune system and the brain appears to be held by astrocytes and endothelial cells (Fontana and Fierz, 1985). In the immune system, CD44 has been shown to allow adhesion of lymphocytes to high endothelial venules, leading to passage of activated cells from the blood into inflamed tissues (Jalkanen et al., 1986). However, the results from Yednock et al. (1992) suggest that CD44 is not directly involved in the binding of lymphocytes to inflamed vessels of EAE rat brain. Nevertheless, our results suggest that CD44 is involved in the cellular interactions controlling lymphocyte retention within the CNS, namely T cell binding to astrocytes.

Our in vitro studies on the adhesion of T cells to astrocytes show that these two cell types are able to bind in an activation-dependent manner. PMA activation of either the H11.1 T cell hybridoma or the astrocyte monolayer resulted in increased adhesive interaction, and binding was maximal when both cell types were activated (Fig. 9 *a*). The combined results of inhibition by hyaluronidase treatment (Fig. 9 *b*), soluble HA (Fig. 9 *c*) and mAb KM81 (Fig. 9, *d* and *e*) indicate that CD44 molecules on both astrocytes and H11.1 T cells participate in cell–cell adhesion, and that the HA-binding domain is crucial for this interaction. In correlation, recent work on HA binding through CD44 shows that IL-5 activated B cells, expressing abundant CD44, can bind to HA-coated dishes (Murakami et al., 1991). Lymphoid cell lines stimulated with PMA also show increased binding of fluorescein-labeled HA (Lesley et al., 1990). However, not all $CD44^+$ cell lines are able to bind HA (Hyman et al., 1991). In all these experiments, it remains to be determined whether a particular variant form of CD44 is responsible for HA binding.

As shown in Fig. 9 *c*, PMA-treated cells were more resistant to inhibition of binding by HA than unstimulated cells. This result suggests that other adhesion molecules or non-HA binding isoforms of CD44 may be involved in the binding between astrocytes and T cells. Thus, leukocyte function accessory molecule-1 (LFA-1) and intercellular adhesion molecule-1 (ICAM-1) are immunological adhesion molecules implicated in cell contacts driving stimulation of astrocytes in inflammation. ICAM-1 has been reported to be increased on the cell surface of astrocytes through γ-IFN stimulation (Hertz et al., 1990), and similarly, treatment of human astrocytes with α-TNF can induce expression of E-selectin, VCAM-1, and ICAM-1 adhesion molecules (Hurwitz et al., 1992). These results might account, at least in

part, for the HA-independent increase of T cell adhesion to activated astrocytes.

In vivo, α-TNF production and activated astrocytes proliferation are observed in EAE lesions, while activated lymphocytes are accumulating in these sites. Results obtained in the present study show that regulation of the CD44 adhesion molecule may play an important role in the pathogenesis of multiple sclerosis. Expression of multiple adhesion molecules by lymphocytes and astrocytes may regulate the persistence of lymphocytes at sites of inflammation within the CNS.

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Note Added in Proof: During the process of this manuscript, Cruz et al. (1993. *Cell Adhesion and Communication*. 1:9-20) reported that a glycoprotein expressed by human fibrous astrocytes and which bound hyaluronate was by biochemical criteria a membrane of the CD44 family.

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