Cytokines Regulate Microglial Adhesion to Laminin and Astrocyte Extracellular Matrix via Protein Kinase C-Dependent Activation of the α6β1 Integrin

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Microglia are highly plastic cells that participate in inflammatory and injury responses within the CNS and that can migrate extensively after activation. Because astrocytes and their extracellular matrix (ECM) form a large part of the CNS parenchyma, we undertook to study the adhesive interactions between microglia and these substrates in vitro. In contrast to oligodendrocyte precursor cells, microglia formed only weak interactions with astrocytes and their ECM. On specific ECM substrates the microglia adhered strongly to fibronectin, vitronectin, and plastic but only weakly to laminin. Microglial adhesion to laminin was increased significantly by the proinflammatory cytokines TNF, IFN-α, and IFN-γ but was decreased by TGF-β1, with the TGF-β1 effect being dominant over the other cytokines. Fluorescence-activated cell sorting (FACS) analysis and immunoprecipitation showed that microglia constitutively express the α6β1 integrin, a well characterized laminin receptor, and that α6β1 expression levels did not change after cytokine treatment. Function-blocking studies showed that microglial adhesion to laminin is mediated entirely by the α6β1 integrin, strongly suggesting that the cytokine regulation of adhesion to laminin is mediated by changes in the activation state of α6β1. Analysis of signaling pathways revealed that activation of α6β1 is mediated by a PKC-dependent mechanism. In light of the evidence that laminin expression is upregulated after CNS injury, the findings suggest that cytokine regulation of microglial adhesion to laminin may play a fundamental role in determining the extent of microglial infiltration into and retention at the site of injury.

Key words: microglia; astrocytes; extracellular matrix; laminin; integrin; cytokine; inflammation; activation; CNS

Cell adhesion to the surrounding extracellular matrix (ECM) or to neighboring cells is a vital function necessary for cell survival, migration, proliferation, and differentiation. Ultimately, it helps to determine the complex spatial cell–cell relationships that are established during development and that continue to be tightly regulated throughout adult life (Adams and Watt, 1993; Sastry and Horwitz, 1996; Hynes, 1999). In the adult the importance of regulation of cell adhesion is nowhere more apparent than in the cells involved in mounting a response to infection or injury, because these cells have to attach and then detach from distinct biological substrates at different stages of the inflammatory process (Dustin and Springer, 1991; Diamond and Springer, 1994). In the CNS a principal cell that fulfills this role is the microglial cell. Microglia behavior is very dynamic and can vary from a quiescent phenotype to an activated, migratory cell that phagocytoses tissue debris and triggers responses involved in the recruitment of other inflammatory cells (Kreutzberg, 1996; Rezaie and Male, 1999).

For microglia and other inflammatory cells to be recruited to a lesion, the cells have to acquire migratory ability. Adhesive interactions with the tissue in which they are responding are critical determinants of migratory behavior (Huttenlocher et al., 1995; Lauffenburger and Horwitz, 1996). In the CNS the astrocytes and their associated ECM form a large part of the potential substrate that microglia will engage during migration; therefore, it is important to understand the molecular basis of the interaction between microglia and astrocytes and their ECM. This becomes more relevant considering that brain injury induces astrocytosis and upregulated expression of many ECM molecules, including fibronectin (Egan and Vijayan, 1991; Pasinetti et al., 1993), laminin (Liesi et al., 1984; Frisen et al., 1995), vitronectin (Sobel et al., 1995; Niquet et al., 1996), and several proteoglycans (Laywell and Steindler, 1991; Bovolenta et al., 1992). In addition, many cytokines also are upregulated after CNS injury, and these molecules can modulate different aspects of glial behavior, including cell adhesion and migration (Feuerstein et al., 1998; Raivich et al., 1999).

Our present understanding of what regulates microglial–astrocyte adhesive interactions is not well defined. Previous studies have been contradictory and show that microglia can display both weak (Gebicke-Haerter et al., 1989; Ben-Hur et al., 1998) and strong attachment to astrocytes (Tanaka et al., 1999; Toku et al., 1999). This variation in cell behavior may be explained by the observation that soluble factors in serum such as lipopolysaccharide (LPS; Gebicke-Haerter et al., 1989) regulate this adhesive interaction (Kloss et al., 2001). The overall objective of our study was to examine the nature of microglial–astrocyte interactions under serum-free conditions, thus reducing the potential of influence by extrinsic factors. Specifically, we wished to (1) examine microglial behavior on an astrocyte monolayer and on astrocyte ECM, (2) investigate microglial adhesion to individual ECM molecules produced by astrocytes in vitro, (3) determine the influence of cytokines on these processes, and (4) elucidate the...
role of specific signal transduction pathways in microglial adhesion interactions.

MATERIALS AND METHODS

**Cell culture.** Mixed glial cultures were prepared as described previously (Milner and ffrench-Constant, 1994) with a technique modified from McCarthy and de Vellis (1980). Briefly, forebrains from postnatal mice (days 0–2) were stripped of meninges, chopped into small chunks, and dissociated in papain before being cultured for ~10 d on poly-d-lysine-coated (Sigma, St. Louis, MO) T75 tissue culture flasks (Falcon, Franklin Lakes, NJ) in DMEM (Sigma) supplemented with 10% fetal calf serum (FCS, Nunc). After establishment of the astrocyte monolayer, the flasks were shaken for 1 h to obtain the loosely attached microglia. Then the microglia were cultured in serum-free N1 medium (DMEM supplemented with N1; Sigma) and used in adhesion assays or for analysis of integrin expression by fluorescence-activated cell sorting (FACS) or immunoprecipitation.

**Preparation of astrocyte extracellular matrix.** Mixed glial cultures were prepared in poly-d-lysine-coated 24-well plates (Nunc, Naperville, IL) as described above and maintained in DMEM containing 10% FCS. Then 1 week after the astrocytes became confluent, the cells were lysed in water for 30 min at 37°C. The excess cell debris was removed by washing the substrate several times in PBS; the remaining ECM substrate was stored in PBS at 4°C and used within 2 d of preparation.

**Antibodies and peptides.** The following antibodies used in immunoprecipitations, FACS analysis, and adhesion assays were obtained from PharMingen (San Diego, CA): the monoclonal antibodies specific for the integrin subunits β1 (H92/5), α1 (H31/8), α4 (R1–2), α5 (5110–27), and α6 (GoH3); the isotype control monoclonal antibody (anti-KLH); and the polyclonal donkey anti-rat phycocyanin-conjugated secondary antibody. The RGD and RGE peptides were obtained from Invitrogen (San Diego, CA).

**Cell surface labeling and immunoprecipitation.** Cell surface molecules were labeled with biotin by removing growth media, washing the cell layer twice with PBS, and then incubating the cells with 0.1 mg/ml NHS-LC-Biotin in PBS (Pierce, Rockford, IL) at 37°C in 5% CO₂ for 30 min. Cell monolayers were washed three times with cell wash buffer (50 mM TRIS-HCl, pH 7.5, 0.15 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂) and harvested with a cell scraper before being washed twice more in suspension. Next the cells were lysed in extraction buffer (cell wash buffer plus 0.5% NP-40, 300 µg/ml PMSF, 1 µg/ml peptatin A, 2 µg/ml aprotinin, and 4 µg/ml leupeptin) for 30 min on ice, followed by trituration and centrifugation at 14,000 rpm at 4°C to remove the insoluble fraction. The supernatants were precleared in a 1 hr incubation with 30 µl of protein G-agarose beads (Roche Diagnostics, Mannheim, Germany) for 2 hr, after which the beads were washed five times in immunoprecipitation buffer (IPB). To the washed beads were added 0.5 M cell lysate. Immunoprecipitations were performed overnight at 4°C on a rotating platform, using the GoH3 monoclonal antibody at 1:100 dilution. The immune complexes were collected by incubation with 30 µl protein G-agarose (Amersham Pharmacia Biotech) per milliliter of protein G-agarose beads (Roche Diagnostics, Mannheim, Germany) for 1 hr. Then the membranes were washed extensively in TBS, and the proteins were identified with the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech) according to the manufacturer’s instructions.

**FACS analysis.** Microglia were cultured in six-well plates (Nunc) in serum-free N1 medium. The presence or absence of the following cytokines: interleukin-3 (IL-3; 1 ng/ml), transforming growth factor-β1 (TGF-β1; 2 ng/ml; R&D), interferon-α (IFN-α; 10^4 U/ml; Invitrogen), and IFN-γ (5 U/ml; 1.6 ng/ml; R&D). After a 12 hr incubation with these cytokines the microglia were scraped off the tissue culture plates and centrifuged; N1 medium blocked in suspension with 2% formaldehyde and 5% normal goat serum for 30 min on ice. Then the microglia were transferred to wells within a round-bottom 96-well plate (Nunc) and incubated with the primary rat anti-mouse monoclonal antibodies (1:100 dilution) for 1 hr on ice. Then the cells were washed twice in the blocking buffer before being labeled with donkey anti-rat-PE conjugate (1:100 dilution; Phar-Mingen) for 1 h on ice. Next the cells were washed twice in the blocking buffer before being resuspended in the fixing solution, 2% formaldehyde in PBS. The fluorescence intensity of the labeled microglia was analyzed with a Becton Dickinson (San Diego, CA) FACScan machine, with 10,000 events recorded for each condition.

**Adhesion assays.** All substrates were prepared by coating the central area of the wells within 24-well tissue culture dishes (Nunc) with 25 µl of ECM solution (10 µg/ml) for 2 hr at 37°C. Bound substrates were washed twice with serum-free N1 medium immediately before the addition of the cells. Microglia (prepared as described above) were centrifuged, resuspended in N1 medium, and applied to the substrates in a 25 µl drop for 1 hr at 37°C. The adhesion assay was stopped by adding 1 ml of DMEM to each well and washing off loosely attached cells. The attached cells then were fixed in 4% paraformaldehyde in PBS for 20 min. Adhesion was quantified by counting all attached cells under phase microscopy; the results were expressed as a percentage of the number of cells adhering under control conditions for each substrate. Within each experiment each condition was performed in duplicate; the results represent the mean ± SEM of three experiments. Statistical significance was assessed by using the Student’s paired t test in which p < 0.05 was defined as statistically significant. Murine laminin-1, bovine fibronectin, and bovine vitronectin (all obtained from Sigma) were used at a coating concentration in PBS. Mixed ECM substrates were prepared by using a coating solution containing 10 µg/ml of each protein for which the level of binding to the substratum was determined as described below. In antibody or peptide-blocking experiments the antibody or peptide was added to the medium that was used to resuspend the cells after their final wash and therefore was present when the cells were added to the substrate and throughout the incubation time. The RGD and RGE peptides were used at a concentration of 0.1 mg/ml, and the monoclonal antibodies Ha2/5 and GoH3 and isotype control were used at a concentration of 5 µg/ml. In experiments designed to investigate the influence of cytokines on microglial adhesion to laminin, the individual cytokines were added to the medium that was used to resuspend the microglia to give the final medium concentrations: IL-3 (1 ng/ml), IL-6 (0.2 µg/ml), IFN-γ (40 ng/ml), TGF-β1 (2 ng/ml), IFN-α (10^5 U/ml), and IFN-γ (5 U/ml = 1.6 ng/ml). In the 12 hr experiments involving cytokines, 0.5 ml of N1 medium containing the cytokine was added around the 25 µl drop of cells that had been allowed to attach for 1 hr. To assess microglial adhesion to the astrocyte monolayer, we grew astrocytes to confluence in 24-well plates before they were shaken mechanically for 1 hr to remove microglia. Then fresh microglia were resuspended in N1 medium containing individual cytokines and/or individual integrin function-blocking reagents, plated on top of the astrocyte monolayer, and cultured in serum-free N1 medium. After 12 hr the medium was removed, 1 ml DMEM was added to the wells, and loosely attached microglia were washed off. Then the cells were fixed in 4% paraformaldehyde in PBS for 20 min, and microglia adhesion was quantified by counting all attached microglia under phase microscopy. In experiments to investigate the influence of individual ECM proteins, molecules, phorbol-12-myristate 13-acetate (PMA; Sigma) was used at concentrations of 1, 3, and 10 nM. Calphostin C (Alexis, San Diego, CA) was used at 10, 30, and 100 nM; H89 (Alexis) was used at 10, 30, and 100 nM. In experiments aimed at investigating the role of de novo protein synthesis in response to inflammatory cytokines, cycloheximide (Sigma) was used at a concentration of 10 µg/ml. Photomicrographs of microglia were taken on a Nikon Diaphot inverted microscope with phase optics.

**Quantification of ECM substrate deposition.** To ensure that equivalent amounts of ECM proteins were deposited on the tissue culture plastic during the mixed ECM substrate preparation, we quantified protein deposition by ELISA. ECM substrates were prepared by coating wells of a 96-well tissue culture plate (Nunc) with 50 µl of ECM solution for 2 hr at 37°C. The ECM solution contained single ECM proteins at a range of concentrations (0–30 µg/ml) or, as described above, a mixture of fibronectin with laminin or vitronectin with laminin, all at a concentration of 10 µg/ml. Then the bound substrates were washed twice with PBS before incubation with a 1% BSA blocking solution in PBS for 30 min at 37°C. The substrates were incubated with primary rabbit antibodies reactive for fibronectin, laminin (both from Sigma), or vitronectin (Chemicon, Temecula, CA) diluted 1:1000 in blocking solution for 1 hr at room temperature, followed by a 1:1000 dilution of anti-rabbit alkaline phosphatase (Sigma) diluted 1:500 in blocking solution for 1 hr at 37°C before being washed four times in PBS. Next the substrates were incubated with the alkaline phosphatase substrate p-nitrophenyl phosphate (Vector Laboratories, Burlingame, CA), and
colorimetric intensity was measured in a microplate reader (Dynex Technologies, Chantilly, VA). In each experiment standard concentration curves for the single ECM proteins fibronectin, vitronectin, and laminin were plotted by using a range of coating concentrations (0–30 μg/ml) with duplicates for each concentration. Absorbance values for each protein within the mixed ECM substrate (in triplicate) then were compared with the absorbance value obtained with a coating solution of 10 μg/ml of the single protein. Over three experiments this showed that the presence of 10 μg/ml laminin in the coating solution did not reduce the deposition of either fibronectin (122 ± 17.7% of the amount of fibronectin deposited with no laminin present) or vitronectin (88.7 ± 15.1% of the amount of vitronectin deposited with no laminin present). In addition, the deposition of laminin was not reduced by the presence of either fibronectin (102 ± 25% of the amount of laminin deposited with no fibronectin present) or the presence of vitronectin (148 ± 32.3% of the amount of laminin deposited with no vitronectin present). Therefore, these results generally do not support the possibility that the effect of laminin is to suppress the binding of vitronectin or fibronectin to the plastic substrate.

RESULTS

Microglia adhere weakly to an astrocyte monolayer

To investigate the adhesive interactions between microglia and astrocytes, we prepared mixed glial cultures from neonatal mouse brain according to the method of McCarthy and de Vellis, as has been described previously (McCarthy and de Vellis, 1980; Milner and ffrench-Constant, 1994). These cultures showed a typical stratification of cell types, with a base layer of astrocytes and a “top” layer consisting of oligodendrocyte precursor cells and microglia. Oligodendrocyte precursor cells and microglia differed fundamentally in their interactions with the astrocyte monolayer (Fig. 1A). Oligodendrocyte precursor cells had a phase-dark appearance indicating a firm attachment to the underlying astrocytes and had several long process extensions. In contrast, microglia showed a rounded-up, phase-bright appearance and were either loosely attached or actually floating around on top of the astrocytes. This differential adhesion of oligodendrocyte precursors and microglia to the astrocyte monolayer is well described and actually forms the basis of an established protocol to separate the two different cell types (Hardy and Reynolds, 1991; Milner et al., 1996; Ben-Hur et al., 1998). In this protocol the mechanical shaking of the culture flasks first detaches microglia from the astrocyte monolayer (1–2 hr) and then much later detaches oligodendrocyte precursors from the astrocytes (15–24 hr). When microglia were plated onto an ECM substrate derived from astrocytes, they showed the same adhesive behavior as on the astrocyte monolayer and were rounded-up, poorly spread cells (Fig. 1B).

In contrast to adhesion to astrocytes, when microglia were plated onto uncoated plastic, they strongly attached and showed a fully adherent and spread morphology within 30 min (Fig. 1C). By comparison, oligodendrocyte precursors did not attach to plastic and remained rounded-up and floating in the medium. This raises the fundamental question as to why microglia, which are such intrinsically adherent cells, attach strongly to a nonspecific plastic substrate but only weakly to the astrocyte monolayer. Considering that adhesive interactions between microglia and astrocytes are likely to be a major factor regulating microglial behavior in vivo, it becomes important to understand the molecular basis of the interaction between these two cell types and to understand how this might be regulated.

Microglia adhere weakly to laminin

After injury to the CNS, astrocytes show upregulated expression of many ECM molecules, and these molecules also are expressed by astrocytes grown in culture. These include the glycoproteins fibronectin (Liesi et al., 1986; Oh and Yong, 1996), vitronectin (Oh and Yong, 1996), and laminin (Liesi et al., 1983; Giotta et al., 1986) and the proteoglycans (Ard and Bunge, 1988). As a first step to investigate whether any of these ECM molecules might regulate microglia–astrocyte interactions, we examined microglial adhesion and spreading characteristics on individual ECM substrates. When 1 hr adhesion assays were performed, microglia adhered well to the control plastic substrate and the ECM substrates fibronectin and vitronectin, but microglial adhesion to laminin was much weaker (see Fig. 3A). In fact, <15% of available microglia adhered to the laminin substrate (12.9 ± 1.4% compared with 53.7 ± 12.1% adhesion to plastic; p < 0.01), and, as seen in Figure 2, the nature of this adhesion was weak, with microglia on laminin showing a phase-bright rounded-up appearance (reminiscent of microglial behavior on astrocytes) in contrast to the well spread phase-dark microglial morphology on the other substrates. Therefore, these experiments showed that laminin is a relatively nonadherent substrate for microglia under these conditions. To investigate further the nature of microglial interactions with ECM molecules, we plated microglia into six-well plates in which part of the surface had been coated with the...
different ECM molecules fibronectin, vitronectin, and laminin. After 24 hr of culture we observed that the areas coated with laminin were almost devoid of any microglia and represented bare patches on the dish, whereas microglia adhered and spread well on fibronectin and vitronectin and uncoated plastic in these longer-term assays. This point is demonstrated in Figure 3C, which shows an interface of laminin and uncoated plastic in which microglia are packed at high density right up to the border of the laminin but do not transgress onto the laminin substrate.

The adhesion assays showed that fibronectin and vitronectin are adhesive substrates for microglia; considering that fibronectin in addition to laminin is present in the ECM synthesized by astrocytes in vitro (Liesi et al., 1986; Oh and Yong, 1996), it is surprising that microglia are not strongly adherent to the astrocyte monolayer and the astrocyte ECM by way of the fibronectin interaction. One possibility is that laminin exerts a dominant anti-adhesive effect on microglial–astrocyte interactions in the mixed glial cultures. To investigate this possibility, we performed adhesion assays by using mixed ECM substrates. To ensure that equivalent amounts of ECM proteins were deposited on the tissue culture plate during the mixed ECM substrate preparation, we quantified protein deposition by ELISA. As described in Materials and Methods, this showed that there was no significant difference in the final amount of the proteins fibronectin or vitronectin deposited on the tissue culture plate when prepared by using either a 10 μg/ml solution of the single protein or a mixed solution containing 10 μg/ml each of fibronectin and laminin or vitronectin and laminin. As shown in Figure 3B, the outcome of this experiment was very clear; although microglia adhered strongly to fibronectin and vitronectin, the presence of laminin reduced microglial adhesion to both of these substrates (from 81.9 ± 7.3 to 12.3 ± 1.3%, p < 0.01 on fibronectin; from 100% control to 14.1 ± 1.7%, p < 0.001 on vitronectin). In other words, laminin exerted a dominant anti-adhesive effect on microglial adhesion.

**Microglia use the α6β1 integrin to attach to laminin**

To investigate whether microglia express any functional cell surface receptors for laminin, we performed analysis by FACS and immunoprecipitation by using the monoclonal antibody GoH3, specific for the well characterized laminin receptor α6β1 integrin. By FACS analysis we determined that microglia express high levels of the α6β1 integrin (Fig. 4A), and this was confirmed by immunoprecipitation (see Fig. 7A). Immunoprecipitation experiments also showed that the α1β1 integrin, another laminin receptor, was not expressed by microglia (data not shown). Once a potential laminin receptor on microglia was identified, the next step was to address the role of this integrin in microglial adhesion to laminin. Short-term microglial adhesion assays were performed for 1 hr in the presence of different integrin function-blocking reagents. As shown in Figure 4B, microglial adhesion to laminin was reduced to baseline levels, both by the β1 integrin monoclonal antibody Ha2/5 (12.7 ± 2.5% of control; p < 0.001) and by the α6 monoclonal antibody GoH3 (14.4 ± 2.3% of control; p < 0.001), but not by RGD peptides or the α4 or α5 monoclonal antibodies. This shows that microglial adhesion to laminin is mediated by β1 integrins and that α6β1 is the major functional laminin receptor expressed by microglia.

**Microglial adhesion to laminin is regulated by cytokines**

The results presented so far indicate something of a paradox; microglia do not attach well to laminin, but they express high
levels of the well recognized laminin receptor α6β1. Previous work has shown that the macrophage, the equivalent cell type outside the CNS, is also weakly adherent to laminin but can be induced to a more adherent phenotype by the presence of IFN-γ (Shaw and Mercurio, 1989). On this basis we set out to determine whether the interaction between microglia and laminin was modified by cytokines also. Adhesion assays were performed in the presence of individual cytokines. In the first instance these assays were performed for 1 hr, and the results showed no significant differences in microglial adhesion to laminin (data not shown). However, when the adhesion assays were extended to 12 hr, clear differences were observed both at the levels of number of cells adherent and also on the microglial morphology. As shown in the summary in Figures 5 and 6, we found that over the 12 hr incubation period only a fraction of the total microglia was adherent to laminin under control conditions (no cytokines), and the cells that did attach were rounded up. Interestingly, TGF-β1 reduced the amount of microglial adhesion to laminin relative to control (no cytokine) (11.2 ± 2.3% compared with 17.9 ± 1.5% control; p < 0.05), and the cells were spread even less than under control conditions. In contrast, the inflammatory cytokines TNF, IFN-α, and IFN-γ significantly increased the number of microglia adherent to laminin (to 44.4 ± 5.7%, p < 0.05; 58.6 ± 7.8%, p < 0.02; and 84.6 ± 5.8%, p < 0.01, respectively, relative to 17.9 ± 1.5% control) and induced a dramatic morphological change so that the microglia became well spread. IL-3 and IL-6 had no noticeable effect. Because these assays showed that microglial adhesion to laminin could be regulated by cytokines in both a positive and a negative direction, additional adhesion assays were performed to determine the effect of combining different cytokines. Because IFN-α and IFN-γ caused the greatest induction of adhesion to laminin and TGF-β1 had the opposite effect, these cytokines were used in combination to determine which effect would predominate. As shown in Figure 6B, TGF-β1 reduced microglial adhesion to laminin both in the presence of IFN-α (9.0 ± 11.1% compared with 52.5 ± 6.8% for IFN-α alone; p < 0.02) and IFN-γ (9.2 ± 4.1% compared with 71.3 ± 5.9% for IFN-γ alone; p < 0.01). In fact, in both of these conditions microglial adhesion to laminin was reduced to the same level as TGF-β1 alone, showing that TGF-β1 exerted a dominant effect over the proinflammatory cytokines. This anti-adhesive effect of TGF-β1 was observed at a range of concentrations from 2.0 to as low as 0.1 ng/ml.

Having determined that several inflammatory cytokines increase the adhesion of microglia to laminin, we wanted to investigate the underlying molecular basis for this change in behavior. There are three main possibilities: first, that the expression level of the α6β1 integrin is regulated; second, that the inflammatory cytokines induce upregulation of another laminin receptor; third, that the activation state of the α6β1 integrin is increased by the inflammatory cytokines, as has been shown before on macro-

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**Figure 4.** Characterization of functional laminin receptors on microglia. A. Purified microglial cultures were analyzed for expression of the α6 integrin subunit by FACS, as described in Materials and Methods. Note that microglia express the α6 integrin subunit. B. Identification of integrins involved in microglial adhesion to different ECM substrates. The 1 hr adhesion assays to fibronectin, vitronectin, or laminin were performed as described in Materials and Methods and in the presence of RGD peptides (control), RGD peptides, isotype control monoclonal antibody, or monoclonal antibodies specific for the β1 (Ha2/5), α4 (R1–2), α5 (5H10–27), or α6 (GoH3) integrin subunits. Adhesion is expressed as a percentage of total microglia adherent to laminin and TGF-β1 reduced the amount of microglial adhesion to laminin (11.2 ± 2.3% compared with 17.9 ± 1.5% control; p < 0.05), and the cells were spread even less than under control conditions. In contrast, the inflammatory cytokines TNF, IFN-α, and IFN-γ significantly increased the number of microglia adherent to laminin (to 44.4 ± 5.7%, p < 0.05; 58.6 ± 7.8%, p < 0.02; and 84.6 ± 5.8%, p < 0.01, respectively, relative to 17.9 ± 1.5% control) and induced a dramatic morphological change so that the microglia became well spread. IL-3 and IL-6 had no noticeable effect. Because these assays showed that microglial adhesion to laminin could be regulated by cytokines in both a positive and a negative direction, additional adhesion assays were performed to determine the effect of combining different cytokines. Because IFN-α and IFN-γ caused the greatest induction of adhesion to laminin and TGF-β1 had the opposite effect, these cytokines were used in combination to determine which effect would predominate. As shown in Figure 6B, TGF-β1 reduced microglial adhesion to laminin both in the presence of IFN-α (9.0 ± 11.1% compared with 52.5 ± 6.8% for IFN-α alone; p < 0.02) and IFN-γ (9.2 ± 4.1% compared with 71.3 ± 5.9% for IFN-γ alone; p < 0.01). In fact, in both of these conditions microglial adhesion to laminin was reduced to the same level as TGF-β1 alone, showing that TGF-β1 exerted a dominant effect over the proinflammatory cytokines. This anti-adhesive effect of TGF-β1 was observed at a range of concentrations from 2.0 to as low as 0.1 ng/ml.

**Figure 5.** The influence of cytokines on microglial adhesion to laminin. Microglia were purified from mixed glial cultures, as described in Materials and Methods, and then plated onto laminin in the presence of no cytokines (A), TGF-β1 (B), IFN-α (C), or IFN-γ (D). Scale bar, 35 μm. Note that after 12 hr in culture the microglia under control conditions were rounded-up but had some small process extensions. In the presence of TGF-β1 the microglia were totally rounded-up and mostly were floating around the dish. In contrast, in the presence of either IFN-α or IFN-γ the microglia became more adherent to laminin and adopted a more flattened and spread morphology.

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*Figure 4.* Characterization of functional laminin receptors on microglia. A. Purified microglial cultures were analyzed for expression of the α6 integrin subunit by FACS, as described in Materials and Methods. Note that microglia express the α6 integrin subunit. B. Identification of integrins involved in microglial adhesion to different ECM substrates. The 1 hr adhesion assays to fibronectin, vitronectin, or laminin were performed as described in Materials and Methods and in the presence of RGD peptides (control), RGD peptides, isotype control monoclonal antibody, or monoclonal antibodies specific for the β1 (Ha2/5), α4 (R1–2), α5 (5H10–27), or α6 (GoH3) integrin subunits. Adhesion is expressed as a percentage of cell adhesion to each ECM substrate under control conditions (RGD peptides); all data points represent the mean ± SEM of three experiments. Note that microglial adhesion to laminin was abolished almost totally by both the anti-β1 and anti-α6 antibodies, but none of the other reagents had any significant effect.
Figure 6. The influence of different cytokines on microglial adhesion to laminin. Microglia were purified from mixed glial cultures, as described in Materials and Methods; then 12 hr adhesion assays to a laminin substrate were performed, and adhesion was quantified by counting the cells under phase microscopy. All data points are expressed as a percentage of cell adhesion to plastic and represent the mean \( \pm \) SEM of three experiments. A. Microglial adhesion assays were performed in the presence of IL-3 (1 ng/ml), IL-6 (0.2 ng/ml), TNF (40 ng/ml), TGF-\( \beta \) (2 ng/ml), IFN-\( \alpha \) (10\(^4\) U/ml), or IFN-\( \gamma \) (5 U/ml = 1.6 ng/ml). Note that TGF-\( \beta \) reduced microglial adhesion to laminin relative to control, whereas the inflammatory cytokines TNF, IFN-\( \alpha \), and IFN-\( \gamma \) all increased the adhesion to laminin. B. Microglial adhesion assays were performed in the presence of TGF-\( \beta \) (2 ng/ml), IFN-\( \alpha \) (10\(^4\) U/ml), IFN-\( \gamma \) (5 U/ml = 1.6 ng/ml), or combinations of IFN-\( \alpha \) + TGF-\( \beta \) or IFN-\( \gamma \) + TGF-\( \beta \) (same concentrations as when used alone). Note that the anti-adhesive effect of TGF-\( \beta \) predominated over the proadhesive effect of the IFNs.

phages (Shaw and Mercurio, 1989). To address the first of these possibilities, we cultured microglia in the presence of the different cytokines for 12 hr and then analyzed the \( \alpha \delta \) integrin expression levels. As shown in Figure 7A, the cytokines produced no detectable change in \( \alpha \delta \) expression levels, as seen in immunoprecipitation, or by FACS analysis. To address the second and third possibilities together, we performed microglial adhesion assays over 12 hr in media containing the cytokines shown to promote microglial adhesion to laminin in the presence or absence of the different integrin function-blocking antibodies. As shown in Figure 7B, the increased microglial adhesion to laminin induced by IFN-\( \alpha \) or IFN-\( \gamma \) was reduced to background level both by the \( \beta 1 \) and the \( \alpha \delta \) function-blocking antibodies (anti-\( \beta 1 \), 2.9 \( \pm \) 1.2%, \( p < 0.01 \) and anti-\( \alpha \delta \), 2.9 \( \pm \) 1.4%, \( p < 0.01 \) compared with 47.6 \( \pm \) 5.8% isotype control for IFN-\( \alpha \); anti-\( \beta 1 \), 3.2 \( \pm \) 0.9%, \( p < 0.01 \) and anti-\( \alpha \delta \), 2.8 \( \pm \) 1.0%, \( p < 0.01 \) compared with 62.7 \( \pm \) 4.6% isotype control for IFN-\( \gamma \)) but was not affected by RGD peptides or monoclonal antibodies against the \( \alpha 4 \) or \( \alpha 5 \) integrin subunits. This strongly suggests that the \( \alpha \delta \beta 1 \) integrin is solely responsible for mediating microglial adhesion to laminin and that regulation of the activation of the \( \alpha \delta \beta 1 \) integrin is most likely the primary mechanism whereby cytokines regulate microglial adhesion to laminin.

These results are very similar to those of Shaw et al. (1990), who showed that macrophage adhesion to laminin increased after exposure to IFN-\( \gamma \), with the response taking up to 8 hr for the maximal effect (Shaw and Mercurio, 1989). Paradoxically, the activation of cell surface receptors is normally a fast process taking place within minutes. One possible explanation for our findings would be that the IFNs stimulate the synthesis of another factor that is responsible for directly activating the \( \alpha \delta \beta 1 \) integrin expressed by microglia. To test this hypothesis, we performed microglia adhesion assays in the presence of IFN-\( \alpha \) or IFN-\( \gamma \) over a 6 hr time course with or without the addition of the protein synthesis inhibitor cycloheximide. As shown in Figure 7D, IFN-\( \alpha \) increased microglial adhesion to laminin over the 6 hr time course, and this was blocked effectively by the presence of cycloheximide (14.2 \( \pm \) 1.5% in the presence of cycloheximide compared with 36.7 \( \pm \) 3.9% cell adhesion with no cycloheximide present; \( p < 0.02 \)). Similar results also were obtained with IFN-\( \gamma \) (data not shown). To address the possibility that the cycloheximide treatment over 6 hr may alter integrin expression by microglia, we quantified integrin expression levels by FACS analysis. In this experiment the microglia were cultured for 6 hr with or without IFN-\( \alpha \), each in the presence or absence of 10 \( \mu \)g/ml cycloheximide. In three separate experiments the presence of cycloheximide had no significant effect on the expression level of either the \( \alpha 5 \) or \( \alpha \delta \) integrin subunits during the 6 hr time course (data not shown). These results support the hypothesis that the ability of the IFNs to increase microglial adhesion to laminin is an indirect effect, involving the synthesis of an intermediate factor that itself increases the activation state of the \( \alpha \delta \beta 1 \) integrin.

**Activation of the \( \alpha \delta \beta 1 \) integrin involves a PKC signaling pathway**

Previous work on macrophages has shown that cell adhesion to laminin is regulated by a change at the level of activation state of the \( \alpha \delta \beta 1 \) integrin and that this increased activation also can be triggered by activators of protein kinase C pathways such as PMA (Mercurio and Shaw, 1988; Shaw et al., 1990). To determine which intracellular pathways in microglia are important for mediating the changes in adhesion to laminin, we performed adhesion assays to determine the effect of (1) PMA stimulation and (2) IFN-\( \alpha \) and IFN-\( \gamma \) stimulation in the presence of specific inhibitors of PKC-mediated and PKA-mediated pathways. As shown in Figures 8 and 9A, microglial adhesion to laminin in 12 hr adhesion assays was enhanced markedly in the presence of the PKC-activating molecule PMA (1 nm, 55.9 \( \pm \) 7.5%, \( p < 0.05 \); 3 nm, 80.3 \( \pm \) 9.8%, \( p < 0.02 \) relative to 19.6 \( \pm \) 3.1% for control), and, as shown in Figure 8B, PMA changed microglial morphology on laminin from phase-bright, rounded-up cells to phase-dark, fully spread cells. This proadhesive effect of PMA also was observed in much shorter 1 hr adhesion assays, suggesting that stimulation of the PKC signaling pathway directly increased the activation state
of the αβ1 integrin. To investigate whether αβ1 activation in response to IFN-α or IFN-γ is dependent on PKC-mediated or PKA-mediated signaling pathways, we used the PKC-specific antagonist Calphostin C (Jarvis et al., 1994) and the PKA-specific antagonist H89 (Savickiene et al., 1999) in 12 hr adhesion assays. As shown in Figure 9B, the ability of both IFN-α and IFN-γ to enhance microglial adhesion to laminin was blocked by the PKC-specific antagonist Calphostin C (15.9 ± 4.1 compared with 51.2 ± 6.4% control for IFN-α, p < 0.05; 18.5 ± 4.0 compared with 65.9 ± 6.1% control for IFN-γ, p < 0.02), but not by the PKA-specific antagonist H89. In addition, the PKC-specific antagonist reversed the enhanced spread morphology of microglia exposed to IFN-γ (Fig. 8D). These results make clear two points: first, that activating PKC pathways (by PMA) leads to activation of the αβ1 integrin on microglia and, second, that activation of microglial αβ1 by IFN-α and IFN-γ involves signaling via the PKC pathway.

Microglial adhesion to astrocytes is regulated by cytokines

To investigate whether cytokines also modulate microglial–astrocyte interactions, we examined mixed glial cultures in serum-free conditions in the presence of different cytokines. After 1 d of culture, clear differences in the morphology of microglia became obvious. Control cultures showed microglia weakly adherent, with some floating in the media. TGF-β1 induced the microglia to become even less adherent, with far more microglia floating in the media. In contrast, the inflammatory cytokines TNF, IFN-α, and IFN-γ induced the microglia to become much more adherent to the astrocytes and changed the microglial morphology from rounded-up, phase-bright cells to phase-dark, well spread cells that have long process extensions (Fig. 10). This effect was quantified by performing adhesion assays over a 12 hr time period and assessing microglial adhesion both to astrocytes and to astrocyte ECM in the presence of the individual cytokines TGF-β1, IFN-α, and IFN-γ. The contribution of the αβ1 integrin to microglial adhesion also was assessed within the same experiment by using the function-blocking antibodies against the α6 and β1 integrin subunits. As shown in Figure 11, the inflammatory cytokines IFN-α and IFN-γ increased microglial adhesion to astrocytes (to 70.2 ± 6.5%, p < 0.02, and 58.6 ± 4.8%, p < 0.02, respectively, compared with 19.8 ± 3.4% under control conditions) and increased microglial adhesion to astrocyte ECM (to 58.2 ± 4.0%, p < 0.05; and 68.7 ± 4.7%, p < 0.05, respectively, compared with 30.4 ± 5.1% under control conditions). In contrast, TGF-β1 reduced microglial adhesion to astrocytes (from 19.8 ± 3.4 control to 12.6 ± 0.8%; p <
and reduced microglial adhesion to astrocyte ECM (from 30.4 ± 3.2% to 17.4 ± 3.2%; p < 0.05). The β1 and α6 antibodies significantly reduced microglial adhesion to astrocytes (from 19.8 ± 3.4% under control conditions to 11.8 ± 2.1%, p < 0.05, and 10.3 ± 1.4%, p < 0.05, respectively) and reduced microglial adhesion to astrocyte ECM (from 30.4 ± 4.7% under control conditions to 9.6 ± 1.1%, p < 0.02, and 11.3 ± 2.4%, p < 0.05, respectively). In addition, the β1 and α6 antibodies in large part abolished the IFN-α-enhanced microglial adhesion to astrocytes (from 70.2 ± 6.5% under control conditions to 15 ± 2.3%, p < 0.01, and 16.3 ± 2.8%, p < 0.01, respectively) and reduced microglial adhesion to astrocyte ECM (from 58.2 ± 4.0% under control conditions to 14.8 ± 3.1%, p < 0.01, and 15.2 ± 2.2%, p < 0.01, respectively). In a similar manner, the β1 and α6 antibodies also inhibited the IFN-γ-enhanced microglial adhesion to astrocytes (from 58.6 ± 4.8% under control conditions to 14.4 ± 3.5%, p < 0.01, and 16.1 ± 2.4%, p < 0.01, respectively) and reduced adhesion to astrocyte ECM (from 68.7 ± 5.1% under control conditions to 15.4 ± 1.6%, p < 0.01, and 17.5 ± 2.4%, p < 0.02, respectively). In these experiments RGD peptides or monoclonal antibodies specific for the α4 or α5 integrin subunits had no significant effect on microglial adhesion to either astrocytes or astrocyte ECM. In conclusion, these experiments show that cytokines influence microglial adhesion to astrocytes and astrocyte ECM in much the same way that they regulate microglial adhesion to laminin; furthermore, they show that the major part of this adhesion and its regulation is mediated by the α6β1 integrin.

**DISCUSSION**

In this study we investigated the nature of adhesive interactions between microglia and astrocytes and their associated ECM in vitro. There were four main conclusions. First, microglia attached weakly to an astrocyte monolayer and astrocyte ECM. Second, microglia attached weakly to laminin relative to fibronectin and vitronectin, and the effect of laminin was dominant, i.e., laminin reduced microglial adhesion to the other ECM substrates. Third, microglial adhesion to laminin and astrocytes was dependent on the activation state of the α6β1 integrin, and this was regulated by cytokines. Fourth, the change in the activation state of the α6β1 integrin was shown to be mediated by PKC-dependent signaling pathways.

**Regulation of microglial adhesion to laminin**

The observation that microglia adhere only weakly to an astrocyte monolayer first was described >10 years ago (Gebicke-Haerter et al., 1989) and represents a surprising finding given that, in the CNS, astrocytes probably represent the most abun-
occurs in microglia within the CNS. The work presented here shows that microglia have a weak affinity interaction with laminin, one of the most abundant components of astrocyte ECM in vitro (Liesi et al., 1983; Giotta et al., 1986). The behavior of microglia on laminin is in contrast to that of other cells of the CNS, including neurons, astrocytes, and oligodendroglial cells, all of which display strong adhesive interactions with laminin (Reichardt and Tomasselli, 1991; Tawil et al., 1993; Letourneau et al., 1994; Milner et al., 1996, 1999). However, the behavior of microglia on laminin is very similar to that of other inflammatory cells such as neutrophils and macrophages, which show no adhesion to laminin under resting conditions but, when activated, dramatically increase their adhesion to laminin. This has been attributed to a regulation at the level of the activation state of the α6β1 integrin (Wei et al., 1997). The evidence presented in this paper strongly suggests that similar regulation occurs in microglia within the CNS.

This work takes on more significance considering the observation that laminin exerted a dominant anti-adhesive effect when presented with fibronectin or vitronectin. The molecular basis for this is unclear but would seem not to involve an active anti-adhesive signal via the α6β1 integrin for two reasons. First, α6β1 integrin function provided a proadhesive signal for microglia, which was lost with the blocking of α6β1 function on laminin; second, blocking α6β1 integrin function on mixed laminin/fibronectin substrates did not result in increased adhesion of microglia (our unpublished observations). In light of the observation that microglia are equally nonadherent to astrocyte ECM, which contains both fibronectin and laminin secreted and organized by astrocytes as a physiological matrix (Liesi et al., 1983; Oh and Yong, 1996), then this interaction takes on greater biological significance. A dominant anti-adhesive effect of laminin already has been described in sensory neurons (Calof and Lander, 1991) and in inflammatory cells (Yanaka et al., 1997). In the latter study the administration of a laminin peptide was shown to reduce the degree of brain injury in an animal model of transient focal cerebral ischemia by reducing leukocyte accumulation and the subsequent extent of postischemic inflammation.

These studies were performed by using microglia derived from the neonatal CNS. Although it is possible that age-related differences may exist, it is accepted widely that neonatal microglia in vitro display many of the properties of microglia in the adult CNS, including the ability to switch from a resting to an activated phenotype (Kloss et al., 2001).

Intracellular signaling within microglia

An interesting finding to emerge from our studies was that, broadly speaking, negative signals appeared to dominate over positive signals in regulating microglial adhesion. First, the anti-adhesive signal from laminin dominated over the proadhesive signal from fibronectin or vitronectin. Second, the anti-adhesive signal from TGF-β1 dominated over the proadhesive signal from the inflammatory cytokines. These observations support the idea...
that biological dampening mechanisms have the upper hand and play an important role in suppressing low-level proinflammatory stimuli, thereby preventing an excessive inflammatory response. The observation that microglial adhesion to laminin can be regulated both in a positive and negative direction provides a useful model in which to investigate the signaling mechanisms. The intracellular signaling pathways used by these cytokines are fundamentally different; TGF-β1 uses predominantly the Smad pathway (Baker and Harland, 1997), whereas the IFNs use the JAK/STAT pathways (Darnell et al., 1994) and other alternative pathways that include PKC (Jun et al., 1995; Yu and Floyd-Smith, 1997). In this study we have begun to dissect out the signaling mechanisms used by the IFNs and have shown that PKC-mediated events are implicated in enhancing microglial adhesion to laminin. Previous reports have shown that PKC signaling also mediates other microglial responses to IFN-γ, including upregulation of nitric oxide synthase (iNOS) and secretion of neurotoxic agents (Klegeris and McGeer, 2000; Kang et al., 2001). In the future it will be important to investigate the intracellular signaling events in microglia in more detail, with two specific questions in mind. First, how and at what point of the intracellular signaling cascade does TGF-β1 inhibit the IFN-mediated process? Second, is there any connection between the STAT and PKC pathways in cascade does TGF-β1 inhibit the IFN-mediated process? Second, is there any connection between the STAT and PKC pathways in response to IFNs? The observation that the microglial response to IFNs takes place over several hours and can be blocked by the protein synthesis inhibitor cycloheximide suggests that IFNs act indirectly, by inducing synthesis of an intermediate factor that increases the activation state of the αβ1 integrin. In ongoing studies we are investigating the nature of the intermediate factor to determine whether this is a soluble factor secreted by microglia or a key intracellular element of the signaling pathway. In addition, we also are examining the adhesive behavior of microglia obtained from STAT-1 null mice in response to stimulation by IFN-α, IFN-γ, and PMA.

Physiological significance of cytokine regulation of microglial adhesion to laminin

For inflammatory cells outside the CNS, it has been shown that cytokines regulate the ability of circulating leukocytes to adhere to the laminin-rich basal lamina of blood vessels during inflammatory conditions, thereby promoting extravasation of leukocytes toward the inflammatory focus (Wei et al., 1997). This raises the important question, what is the function of enhancing microglial binding to laminin in the CNS? One possibility is that microglia now could attach more strongly to the basal lamina around blood vessels and then migrate along vessels to reach their target site of inflammation. In light of the extensive evidence that CNS injury is accompanied by the upregulation of laminin (Liesi et al., 1984; Giffordristos and David, 1988; Yamamoto and Kawana, 1990; Alonso and Privat, 1993; Logan et al., 1994; Frisen et al., 1995; Stichel et al., 1999), an alternative possibility is that cytokines influence the ability of microglia to adhere to and enter the laminin-rich injury site by regulating the activation state of the αβ1 integrin expressed by microglia in vivo (Terpe et al., 1994; Kloss et al., 1999). On the basis of our data, the ability of microglia to enter the laminin-rich injury site will be regulated by the balance of cytokines present in this region. The proinflammatory cytokines TNF, IFN-α, and IFN-γ will promote increased microglial adhesion to laminin, allowing more microglia to enter and be retained at the injury site to phagocytose cell debris and secrete potential regeneration factors. In contrast, the elevated levels of TGF-β1 observed at the injury site (Pasinetti et al., 1993; Logan et al., 1994) will reduce microglial adhesion to laminin and thus restrict microglial entry into the laminin-rich area, leading to increased scar formation because of the persistence of cell debris. In support of this model, a previous study on injured rat brain showed that function-blocking antibodies against TGF-β1 significantly increased microglial cell number at the site of injury (Logan et al., 1994) and also attenuated glial scar formation, thus providing an environment more conducive to neural regeneration.

Several lines of evidence suggest that failure of effective regeneration in the CNS might be attributable to an inadequate microglial/macrophage response after injury (Perry et al., 1995; Lazarov-Spiegler et al., 1998). First, macrophage recruitment and activation in the CNS are not just delayed but also spatially restricted when compared with the PNS (Perry et al., 1987; George and Griffin, 1994). Second, in mice that have a deficient macrophage response the nerve regeneration is delayed (Ludwin and Bisby, 1992). Third, transplantation of activated microglia and macrophages into lesioned spinal cord leads to enhanced neurite outgrowth (Prewitt et al., 1997; Rabchevsky and Streit, 1997). Taken together, this evidence suggests that one way of promoting more effective regeneration in the CNS would be to stimulate the macrophage/microglia response after injury. This could be attained in part by promoting microglial adhesion and entry into areas of CNS damage. On the basis of the results described in this paper, microglial adhesion and migration into the laminin-rich injury site would be promoted by stimulating activation of the αβ1 integrin. This could be achieved either by regulating the balance or action of cytokines, for instance by blocking TGF-β1 activity (as has been shown already), or by pharmacologically increasing the activation state of the αβ1 integrin on microglia by specific reagents. Future experiments will address the value of this approach.

REFERENCES


