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A Role in Migration for the αvβ1 Integrin Expressed on Oligodendrocyte Precursors

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Myelination of the CNS requires the migration of oligodendrocyte precursors throughout the CNS from restricted regions within the ventricular and subventricular zones. In light of the significant effects of cell–extracellular matrix (ECM) interactions on cell migration in other developing systems, we have analyzed the role of integrins in oligodendrocyte precursor migration. We have shown previously that oligodendrocyte precursors *in vitro* express a limited repertoire of integrins, including α6β1, αvβ1, and αvβ3, and that differentiation is associated with downregulation of αvβ1 and upregulation of αvβ5. Using a migration assay based on the movement of cells away from an agarose drop containing a high-density cell suspension, we find that RGD peptides (that block αv but not α6 integrins) and anti-β1 antibodies block migration on an astrocyte-derived ECM, whereas anti-β3 antibodies have little effect. These results suggest that αvβ1 but not α6β1 plays a role in oligodendrocyte precursor migration, and this is confirmed by the use of blocking monoclonal antibodies that distinguish these two integrins. In keeping with the results of others, we find that differentiated oligodendrocytes lose migratory potential and that the timing of this loss correlates with downregulation of αvβ1. Taken together with the work of others showing that ECM ligands for αvβ1 are expressed within the CNS, we propose that this integrin plays a significant role in the migration of oligodendrocyte precursors *in vivo* and that its downregulation during differentiation could be an important factor regulating the migratory phenotype of these cells.

Key words: oligodendrocyte; integrin; extracellular matrix; platelet-derived growth factor; differentiation; migration; astroglial matrix; vitronectin

The importance of cell migration during development is well illustrated in the CNS, where neurons arise in the ventricular zone and then migrate away to their final destinations (Rakic, 1972). Another cell type that shows extensive migration is the oligodendrocyte precursor, or O-2A progenitor cell. This cell type arises in the ventricular and subventricular zones of the developing CNS (Paterson et al., 1973) and migrates during development to produce the widespread distribution of differentiated oligodendrocytes seen in the mature animal. Direct evidence for this migration has come from two sets of *in vivo* studies. First, cells labeled with a *lac-Z* reporter gene while in the subventricular zone subsequently migrate into the developing cortex and subcortical white matter and form oligodendrocytes (Levison and Goldman, 1993). Second, migration is seen when oligodendrocyte precursors are transplanted into myelin-deficient or normal mice (Lachapelle et al., 1984, 1994).

Previous studies examining the molecular mechanisms regulating oligodendroglial migration have focused on the role of growth factors. These studies have demonstrated that platelet-derived growth factor (PDGF) promotes oligodendrocyte precursor migration in culture and that withdrawal of PDGF is accompanied by differentiation and loss of migratory activity (Small et al., 1987; Noble et al., 1988; Armstrong et al., 1990). Interactions between cells and the extracellular matrix (ECM), however, can also provide instructive signals for many aspects of cell behavior, including survival, proliferation, differentiation, and migration (Streuli et al., 1991; Adams and Watt, 1993), and may therefore play a role in regulating oligodendrocyte precursor migration. Integrins are one important family of ECM receptors. These are cell-surface heterodimeric molecules consisting of α and β subunits made from at least 14 α and 9 β mammalian integrin subunits, which may combine in various ways to confer distinct ligand binding and cell signaling properties (Hemler, 1990; Ruoslahti, 1991; Hynes, 1992; Hynes and Lander, 1992; Diamond and Springer, 1994). Within the nervous system, integrins play a role in the migration of both neural crest cells (Bronner-Fraser, 1986) and neuroblasts in the developing chick tectum (Galileo et al., 1992), but their role in glial migration remains unknown.

To investigate the roles of cell–ECM interactions in regulating oligodendrocyte behavior, we characterized the integrins expressed by oligodendrocytes and their precursors *in vitro*. These studies show that oligodendroglial cells express a limited repertoire of integrins comprising α6β1 and several αv integrins (Milner and ffrench-Constant, 1994; Shaw et al., 1996). αv integrin expression is developmentally regulated: αvβ1 is lost and αvβ5 upregulated with differentiation (Milner and ffrench-Constant, 1994). In the current study, we have investigated the function of these integrins in oligodendrocyte precursor migration. We show

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that ECM substrates recognized by oligodendroglial integrins, including laminin, fibronectin, and vitronectin, promote migration. whereas collagen, not recognized by oligodendroglial integrins, does not. In addition, we find that blocking integrin function inhibits migration on an astrocyte-derived ECM and that the $\alpha v\beta 1$ integrin, but not $\alpha 6\beta 1$ or $\alpha v\beta 3$, plays the dominant role in integrin-mediated migration of oligodendrocyte precursor cells on this substrate.

MATERIALS AND METHODS

Cell culture. Purified oligodendrocyte precursors were obtained as described previously (Milner and ffrench-Constant, 1994), using a technique modified from McCarthy and De Vellis (1980). Briefly, primary cultures were established from rat or mouse neonatal forebrain cells obtained by dissociating cortices in papain, and they were grown for ~10 d in DMEM supplemented with 10% fetal calf serum (FCS) (Globepharm) before being shaken overnight to separate the loosely attached oligodendrocyte precursors ("top cells"). The cells were purified further by removing contaminating microglia using selective adhesion to nontissue culture-treated plastic. The remaining cell suspension was then centrifuged and resuspended in Sato media [DMEM supplemented with bovine insulin (Sigma, St. Louis, MO) (5 μg/ml), human transferrin (Sigma) (50 μg/ml), BSA V (Sigma) (100 μg/ml), progesterone (Sigma) (6.2 ng/ml), putrescine (Sigma) (16 μg/ml), sodium selenite (Sigma) (5 ng/ml), T3 (Sigma) (400 ng/ml), T4 (Sigma) (400 ng/ml), L-glutamine (Sigma) (4 mM), penicillin and streptomycin (Sigma)] containing 0.5% FCS, and secondary cultures were established by plating the cells onto poly-ornithine-coated six-well plates (Nunc, Naperville, IL) or 100 mm petri dishes (Nunc). The purity of the resulting cell suspension was assessed by morphology, with process-bearing oligodendroglia distinguished from fibroblast-like astrocytes and microglia, and was >95% oligodendroglial cells (oligodendrocytes and precursors) at day 1. In these secondary cultures, precursor cells constitutively differentiate into oligodendrocytes. This was confirmed by immunostaining with a monoclonal antibody (Ranscht et al., 1982) against the oligodendrocyte-specific marker galactocerebroside (GalC) (Raff et al., 1978). By immunocytochemistry, the oligodendroglial cells in these cultures were <10% GalC+ after 4 hr (i.e., >90% precursor cells) but >80% GalC+ at day 7 and >95% GalC+ by day 10. Astrocytes were obtained by removing all top cells from a wholebrain flask and using the basal layer of astrocytes left behind. Mouse fibroblasts were obtained from meninges, as described previously (Milner and ffrench-Constant, 1994), and then cultured in DMEM supplemented with 10% FCS and L-glutamine (Sigma)(4 mM) and penicillin and streptomycin (Sigma).

Antibodies. The antibodies used in immunoprecipitations and adhesion and migration assays were generous gifts of the following: (1) monoclonals: GoH3 (rat IgG1, anti-α6) from Dr. Arnoud Sonnenberg, Amsterdam, Holland (Sonnenberg et al., 1987), 9EG7 (rat IgG2a, anti-β1) from Dr. Dietmar Vestweber, Freiburg, Germany (Lenter et al., 1993), and F11 (mouse IgG1, anti-β3) from Dr. Michael Horton, University College London, London, UK (Helfrich et al., 1992b); and (2) polyclonals: anti-ECMR (GP140) from Dr. Caroline Damsky, San Francisco, CA (Knudsen et al., 1981; Damsky et al., 1982), and anti-αv from Dr. Guido Tarone, Torino, Italy (Hirsch et al., 1994). The anti-αvβ3 antiserum and RGD and RGE peptides were obtained from Life Technologies (Paisley, Scotland). Additional purified GoH3 was obtained from Serotec (Oxford, UK), and 9EG7 was obtained from PharMingen (San Diego, CA).

The preparation and characterization of the function-blocking anti-β1 antibody will be described elsewhere (G. Edwards and C. Streuli, unpublished observations) and is summarized briefly here. α4β1 integrin was affinity-purified under nondenaturing conditions from whole mouse embryos using sepharose-conjugated PS/2, a rat monoclonal antibody specific for mouse α4 integrin. The purified integrin was used to immunize rabbits, and IgG was isolated from the resulting sera. This antibody specifically recognized both α4 and β1 integrin subunits in Western blotting and immunoprecipitation experiments.

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 labeling with 0.1 mg/ml NHS-LC-Biotin in PBS (Pierce, Rockford, IL) at 37°C in 7.5% CO_2 for 30 min. Cell monolayers were then washed three times with cell wash buffer (50 mM Tris-HCL, pH 7.5, 0.15 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$) and harvested with a cell scraper before being washed twice more in suspension. Cells were then lysed in 1% NP40 extraction buffer (cell wash buffer plus 300 μg/ml PMSF, 1 μg/ml pepstatin 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 then were precleared by two sequential 2 hr incubations with 30 μl of protein A-sepharose (Pharmacia, Piscataway, NJ) and 4 μl of nonimmune rabbit serum/ml of cell lysate. Immunoprecipitations were carried out overnight at 4°C on a rotating platform using 1 μl of rabbit antisera/250 μl of cell lysate. Where rat monoclonal antibodies were used (GoH3 and 9EG7), rabbit anti-rat antisera (Nordic, Capistrano Beach, CA) was also added at 1:250 to the tube. The immune complexes were collected by incubation with 30 μl of protein A-sepharose beads for 2 hr, after which time the beads were washed five times in immunoprecipitation wash buffer [identical to the cell wash buffer except for a higher salt concentration (0.5 M NaCl) and the addition of 0.1% Tween 20]. Integrins then were separated from the beads by boiling in nonreducing SDS sample buffer for 5 min before being analyzed by SDS-PAGE on a 7.5% resolving gel and 4% stacking gel under nonreducing conditions. Proteins were then electroblotted for 3 hr onto nitrocellulose (Hybond-C, Amersham, Arlington

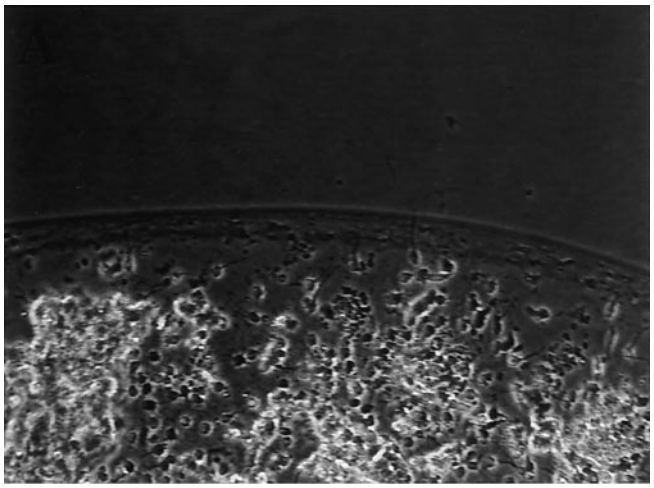

Figure 1. The effect of PDGF on the migration of oligodendrocyte precursors. Cells were resuspended at high density in agarose, as described in Materials and Methods, and then plated as small drops onto poly-DL-ornithine-coated plastic and cultured in the absence (*A*) or presence (*B*) of PDGF (5 ng/ml). Note that PDGF promotes the migration of oligodendrocyte precursors, with no migration observed in the absence of this growth factor.

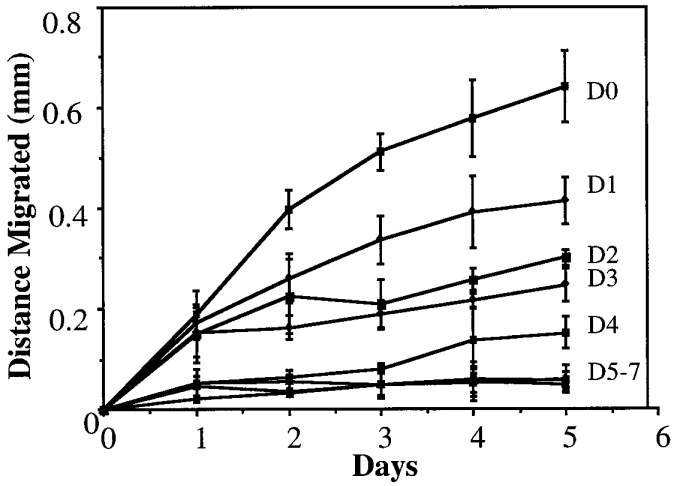

Figure 2. The relationship between differentiation and migration in oligodendrocyte precursor cells. Cells were resuspended at high density in agarose, as described in Materials and Methods, and then plated as small drops onto poly-DL-ornithine-coated plastic and grown in defined medium alone, without PDGF. Cells were then allowed to differentiate for 0–7 d before PDGF was added to promote migration, and migration was measured for the following 5 d. Data from three experiments are shown. Note that the more the cell populations are allowed to differentiate before the addition of PDGF (most differentiated at day 7, least at day 0), the less they migrate in response to PDGF.

Heights, IL), blocked overnight with 3% BSA in TBS (10 mM Tris-HCL, 0.15 NaCl, pH 8.0) containing 0.1% Tween 20, and detected with streptavidin-HRP (ECL detection system, Amersham) for 1 hr according to manufacturer's instructions.

Adhesion assays. All substrates were prepared by coating small areas of a 90 mm bacteriological grade plastic petri dish with 50 μl of ECM solution (10 μg/ml) or poly-DL-ornithine (5 μg/ml) for 2 hr at 37°C. Before the addition of cells, all substrates were blocked with 50 μl of heat-inactivated (5 min at 80°C) 0.3% BSA (BSA-fraction V, Sigma) for 30 min to prevent nonspecific binding to substrates. Substrates were then washed twice with Sato media immediately before addition of the cells. Oligodendrocyte precursors, freshly isolated from rat or mouse primary forebrain cultures as described above, were centrifuged, resuspended in Sato media, and applied to the substrates in a 50 μl drop for 30 min at 37°C. The adhesion assay was stopped by adding 10 ml of DMEM to the petri dish and washing off loosely attached cells. The attached cells were then fixed with 4% paraformaldehyde in PBS for 20 min. Adhesion was quantified by counting all attached cells under phase microscopy. Murine laminin, bovine fibronectin, and bovine vitronectin were all obtained from Sigma and diluted to the required concentration in PBS. In antibody-blocking experiments, the antibody was added to the medium used to resuspend the cells after their final wash and therefore was present when the cells were added to the substrate and throughout the experiment. The polyclonal anti-ECMR antibody was used at a dilution of 1:400. The monoclonal 9EG7 antibody was used at a concentration of 5 μg/ml, and GoH3 was used at a range of concentrations from 0 to 10 μg/ml.

Cell migration assay. Cell migration was quantified by measuring the extent of migration from agarose drops using a modification of the method described by Varani et al. (1978). Oligodendrocyte precursors were obtained as described above and resuspended at 40×10^6 cells/ml in Sato media containing 10% FCS and 0.3% low melting point agarose (Sigma) maintained at 37°C to prevent setting of the agarose; 1.5 μl drops of the cell suspension were applied to the center of wells within a 24-well tissue culture dish (Nunc), which was then placed at 4°C for 15 min to allow the agarose to solidify. Three different sets of substrates were used in these experiments. For experiments using astroglial matrix (AGM), the matrix was prepared in 24-well plates before the addition of the drop by removing growth media from mixed glial cultures and adding 1 ml of water per well for 2 hr at 37°C. The lysed cell material was then removed with three washes of PBS, before the AGM was stored in PBS at 37°C. Agarose drops were placed onto this matrix after removal of the PBS and covered with 0.4 ml of serum-free Sato media after cooling. For experiments using poly-DL-ornithine substrates, the plastic was coated as described above, washed with water, and dried, after which the agarose drop was added and covered with 0.4 ml of serum-free Sato media after cooling. For purified ECM substrates, drops were plated directly onto tissue-culture plastic, and the cooled drop was surrounded by 50 μl of Sato media containing 10 μg/ml of the chosen ECM molecule and incubated for 2 hr at 37°C. After this incubation, 0.35 ml of media was added. With the exception of the first set of experiments to examine the effect of PDGF on oligodendrocyte precursor migration, PDGF was always present at 5 ng/ml. Cell migration was measured at daily intervals for 1–5 d using a phase microscope with a calibrated graticule in the eyepiece, in which the width of one grid square represented 100 μm actual distance at a magnification of 10×. Cells migrate out to form a uniform corona around the drop. At any one time point, the distance between the edge of the drop and the leading edge of migrating cells within the corona was recorded on four sides of the drop. The few individual cells that had migrated ahead of the corona were not included in the measurement. Within single experiments, each condition was tested in duplicate or triplicate. The mean migration was calculated for each experiment, and results were expressed as mean ± SEM. Statistical significance was assessed by using the Students paired *t* test, in which $p < 0.05$ was defined as statistically significant. Blocking antibodies and peptides were added to the wells immediately after addition of the surrounding media. Antibodies were added only once, whereas RGE and RGD peptides were added at daily intervals. Antibodies were used at the following dilutions/concentrations: anti-ECMR antiserum, 1:400; anti-αvβ3 antiserum, 1:100; anti-β1 antisera, 60 μg/ml; 9EG7, 5 μg/ml; and GoH3, 5 μg/ml. RGE and RGD peptides were used at a concentration of 0.1 mg/ml. Photomicrographs of the agarose drop assays were taken on a Nikon Diaphot inverted microscope using phase optics.

RESULTS

Oligodendrocyte precursors migrate radially from an agarose drop in response to PDGF

To measure the migration of oligodendrocyte precursors, we have modified the technique described by Varani et al. (1978). This assay measures cell migration away from a high-density population of cells contained in an agarose drop. Purified populations of oligodendrocyte precursor cells were resuspended in a 1.5 μl drop containing 0.3% low melting point agarose and plated onto a poly-DL-ornithine substrate, as described in Materials and Methods. A defined media of serum-free Sato medium (see Materials and Methods) was then added to the culture. Within 2 hr of plating, oligodendrocyte precursors had begun to migrate out of the drop, and they continued to migrate radially for a number of days, producing a uniform corona of cells (Fig. 1.). Migration was quantified daily by measuring the distance between the leading edge of the corona and the edge of the agarose drop. As expected from previous studies using different assays (Noble et al., 1988; Armstrong et al., 1990), PDGF promoted oligodendrocyte precursor migration in this assay. Indeed PDGF seemed to be required for migration; in the presence of PDGF, oligodendrocyte precursors migrated distances in excess of 1 mm during a 5 d period, whereas in the absence of PDGF there was no migration (Fig. 1).

PDGF also acts as a mitogen for oligodendrocyte precursor cells (Noble et al., 1988; Raff et al., 1988), and time-lapse analyses showed cell division as oligodendrocyte precursors migrated away from the drop, confirming that PDGF has both mitogenic and migration-enhancing properties. This raises the possibility that the movement of oligodendrocyte precursors away from the drop may be a consequence of cell division, with increasing cell density causing passive movement, rather than being attributable to active cell migration. To separate these two effects of PDGF, migration assays were performed in the presence of the mitotic inhibitor aphidicholin, which inhibits oligodendroglial cell division (McKinnon et al., 1993). The maximal extent of oligodendrocyte

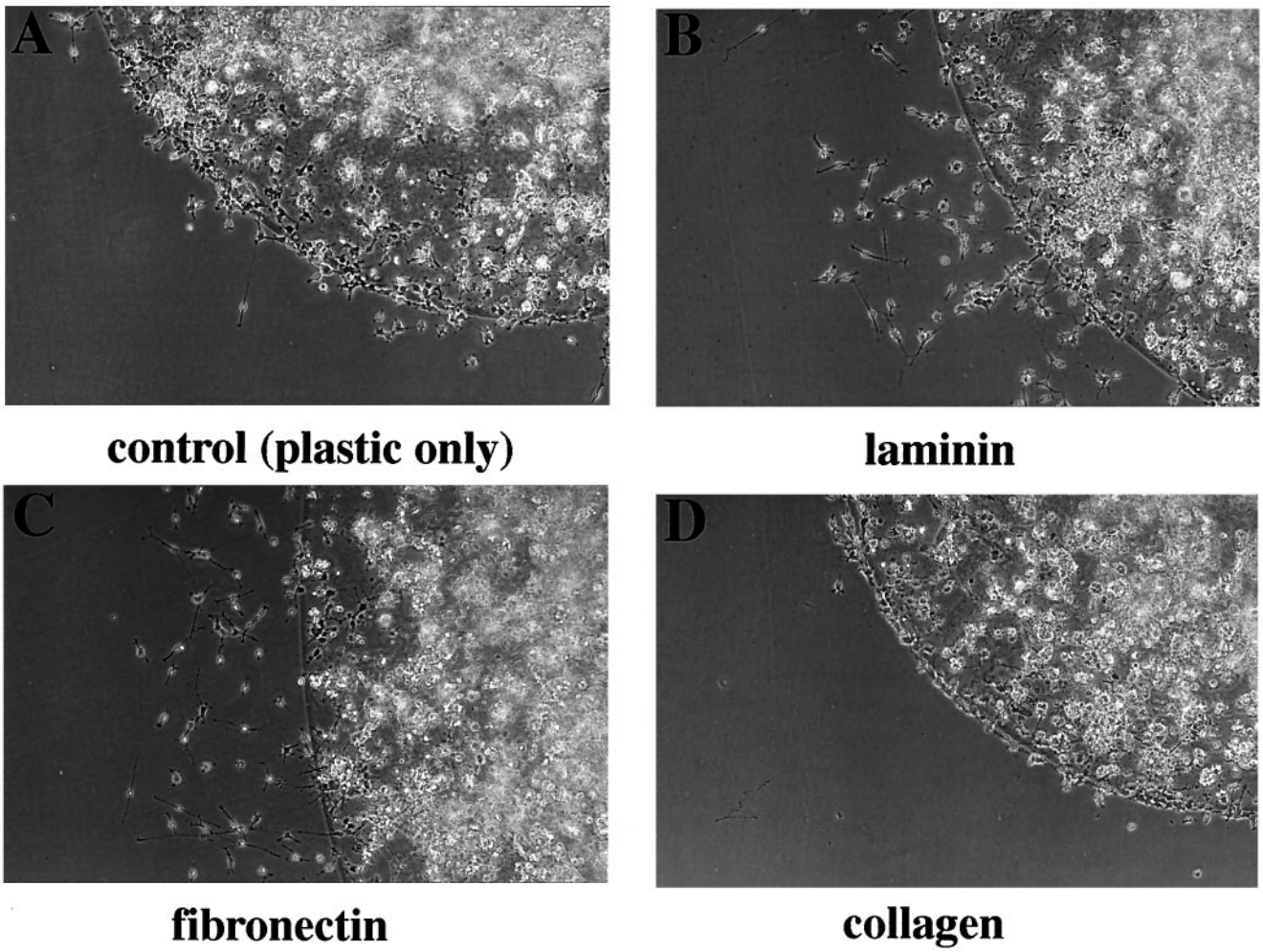

Figure 3. The migration of oligodendrocyte precursors on different ECM substrates. Cells were resuspended at high density in agarose, as described in Materials and Methods, and then plated as small drops onto uncoated tissue-culture plastic. The ECM substrate was then added, as described in Materials and Methods. *A*, Control with no ECM; *B*, laminin; *C*, fibronectin; *D*, collagen. Note that after 2 d, cells on laminin and fibronectin had migrated further than the uncoated plastic control. Cells on collagen, in contrast, had migrated less than the control and have lined up around the periphery of the agarose drop.

precursor migration under these conditions was the same as that seen in the absence of aphidicholin (data not shown). This confirms that the dispersal of oligodendrocyte precursors in the agarose drop assay reflects cell migration.

Differentiation of oligodendrocyte precursors reduces migratory potential

Previous studies show that the oligodendrocyte precursor is a migratory cell, whereas differentiated oligodendrocytes possess no migratory potential (Small et al., 1987; Noble et al., 1988). To examine the timing of this loss of migratory ability, we took advantage of the observation that oligodendrocyte precursors grown in defined medium without added mitogens differentiate constitutively into oligodendrocytes (Temple and Raff, 1985). This differentiation occurred over a 7 d period in our cultures, after which the majority of cells in our cultures have stopped dividing and have differentiated into GalC+ oligodendrocytes (Milner and ffrench-Constant, 1994). Populations of oligodendrocyte precursors in agarose drops were allowed to differentiate for various times before the addition of PDGF. On successive days after plating, PDGF was then added to the media to stimulate migration, and the extent of migration was measured for the following 5 d. As shown in Figure 2, oligodendrocyte precursors maintained from the start (day 0) in PDGF show a high level of migration throughout the 5 d period. Cell populations cultured in defined medium alone without PDGF for 3 d (day 3) showed reduced migratory ability once PDGF was added. Cell populations cultured in defined medium alone without PDGF for 7 d (day 7), allowing almost complete differentiation, showed virtually no migration on addition of PDGF. Therefore, as expected from previous studies (Small et al., 1987; Noble et al., 1988), there was an inverse correlation between the extent of differentiation within the population and the ability to migrate, with no migration seen in differentiated oligodendrocytes. PDGF was included in all further studies at the time of plating so that the assay could be used to measure migration of undifferentiated oligodendrocyte precursors.

ECM molecules promote oligodendrocyte precursor migration by integrin-dependent mechanisms

To assess the ability of oligodendrocyte precursor integrins to promote migration, we examined migration on three ECM ligands—laminin, fibronectin, and vitronectin—recognized by these integrins (Cheresh and Spiro, 1987; Cheresh et al., 1989; Bodary

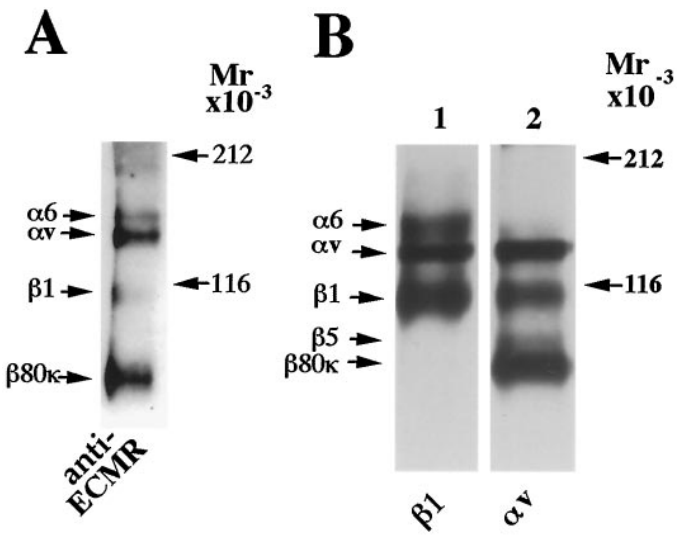

Figure 4. Immunoprecipitations of integrins from rat oligodendrocyte precursors to illustrate the specificity of the polyclonal antibodies used. Immunoprecipitations of biotin-labeled cell-surface proteins were performed, as described in Materials and Methods, with the anti-ECMR antiserum (*A*), anti-β1 antiserum (*B, lane 1*), or anti-αv antisera (*B, lane 2*). After this, the proteins were separated on nonreducing gels. Note that the anti-ECMR antiserum immunoprecipitates a repertoire of bands corresponding to all of the integrins expressed by oligodendrocyte precursors ($\alpha6\beta1$, $\alpha v\beta1$, and $\alpha v\beta80$k). The anti-β1 antiserum recognizes the β1 subunit in association with two α subunits corresponding to α6 and αv, but it does not cross-react with β80k.

and McLean, 1990), and on one ECM ligand, type-1 collagen, for which oligodendrocyte precursors lack any recognized integrin receptors. An example of these assays is shown in Figure 3, which shows that laminin and fibronectin promote migration after 2 d, whereas collagen inhibits migration relative to the tissue-culture plastic control. Combined data from four separate experiments demonstrate that laminin, fibronectin, and vitronectin all increased the rate of migration as compared with uncoated plastic. Laminin and fibronectin were the most effective, promoting the extent of migration after 2 d to 191.9 ± 18.4% ($p < 0.002$) and 188.5 ± 23.6% ($p < 0.02$) of the uncoated plastic control, respectively, with vitronectin promoting migration to 132.2 ± 12.6% of the uncoated plastic control. In contrast, migration on a collagen substrate was reduced to 48.4 ± 4.8% ($p < 0.002$) of the control. On uncoated tissue-culture plastic, migration of cells is initially slower than on laminin or fibronectin, but the cells then increase speed and migrate at a constant rate for the duration of the assay.

Having established that ECM molecules recognized by oligodendroglial integrins promote migration, we next confirmed the role of integrins in this migration by blocking the function of all oligodendroglial integrins using the anti-αv and anti-β1 function-blocking antibody anti-ECMR (Knudsen et al., 1981; Damsky et al., 1982). Immunoprecipitations performed with this antibody on oligodendrocyte precursors grown in secondary culture for 2 d show that anti-ECMR immunoprecipitates the same pattern of bands as a combined αv and β1 immmunoprecipitation, with bands running at 150 (α6), 140 (αv), 110 (β1), and 80 kDa ($\beta3/\beta80$k) (Fig. 4*A*). This confirms that the anti-ECMR antibody recognizes all of the integrins expressed by oligodendrocyte precursors (Milner and ffrench-Constant, 1994). As shown in Figure 5, the anti-ECMR antibody significantly inhibited oligodendrocyte precursor migration on all substrates tested, including laminin (36.7 ± 1.93% of control), fibronectin (25.26 ± 6.00% of control), and vitronectin (11.78 ± 9.20% of control) ($n = 3$; $p < 0.001$ for all substrates).

An astrocyte-derived ECM promotes oligodendrocyte precursor migration

In addition to examining migration on purified ECM substrates, oligodendrocyte precursor migration was also examined on astroglial matrix (AGM), an ECM secreted by cortical astrocytes that may mimic the physiological ECM within CNS white matter (Cardwell and Rome, 1988b; Malek-Hedayat and Rome, 1994). This substrate provides a model of that encountered by migrating oligodendrocyte precursors *in vivo* because astrocytes form the normal neighbors of the precursors and axons within white matter tracts. In this assay, the AGM substrate also promoted extensive migration of oligodendrocyte precursors (Fig. 6). All subsequent experiments examining integrin function in oligodendrocyte precursor migration over ECM substrates were therefore performed on this substrate.

RGD peptides and anti-β1 antibodies inhibit oligodendrocyte precursor migration

To establish whether any one of the integrins expressed by oligodendrocyte precursors plays a dominant role in the migration on AGM, we performed function-blocking experiments using RGD peptides, anti-β1 antiserum, and two different anti-β3 antibodies. As discussed in Materials and Methods, the anti-β1 antiserum was raised against purified $\alpha4\beta1$ and recognizes both the α4 and β1 subunits. The α4 integrin is not expressed on oligodendroglial cells, and so the antiserum will only recognize β1-containing integrins on these cells. The specificity of the anti-β1 antiserum is shown in Figure 4*B*, which demonstrates that it immunoprecipitates the β1 subunit and the two associated α subunits (αv and α6) from oligodendrocyte precursor cells, but not β5 or $\beta3/\beta80$k.

For these blocking experiments, migration assays were carried out for 2 d. As shown in Figures 6 and 7, the control RGE peptide had no effect on the extent of migration, whereas the RGD peptide reduced migration to ~55% of the control ($p < 0.002$). The anti-β1 antiserum reduced the extent of migration to ~45% ($p < 0.001$). Two different antibodies against the β3 integrin subunit were used: an anti-$\alpha v\beta3$ antisera (Suzuki et al., 1986; Tawil et al., 1994), which had no significant effect on migration, and the function-blocking monoclonal antibody F11 (Helfrich et al., 1992a), which also had no effect. When both anti-β1 and anti-$\alpha v\beta3$ antisera were included, cell migration was reduced to ~35% of the control ($p < 0.01$), which was not significantly greater than the blockade mediated by anti-β1 antiserum alone.

Inhibition of migration by the RGD peptide or anti-β1 antiserum was not associated with any change in morphology of the oligodendrocyte precursors. This suggests that the block to migration is not a consequence of differentiation, which is associated with a loss of migratory potential, as shown earlier. To confirm this, cells were allowed to migrate from the agarose drops for 1 d without any blocking agent present and then blocked with either anti-β1 antiserum or RGD peptide for 2 d, after which time the block was removed (Fig. 8). The subsequent rate of migration (as assessed by the slope of the graph showing distance traveled vs time) of these cells was compared with cells under control conditions that had not been exposed to blocking agents. If differentiation was induced by the blocking agents, then migration after removal of the block would be slower than that under control conditions. As shown in Figure 8, however, cells whose migration

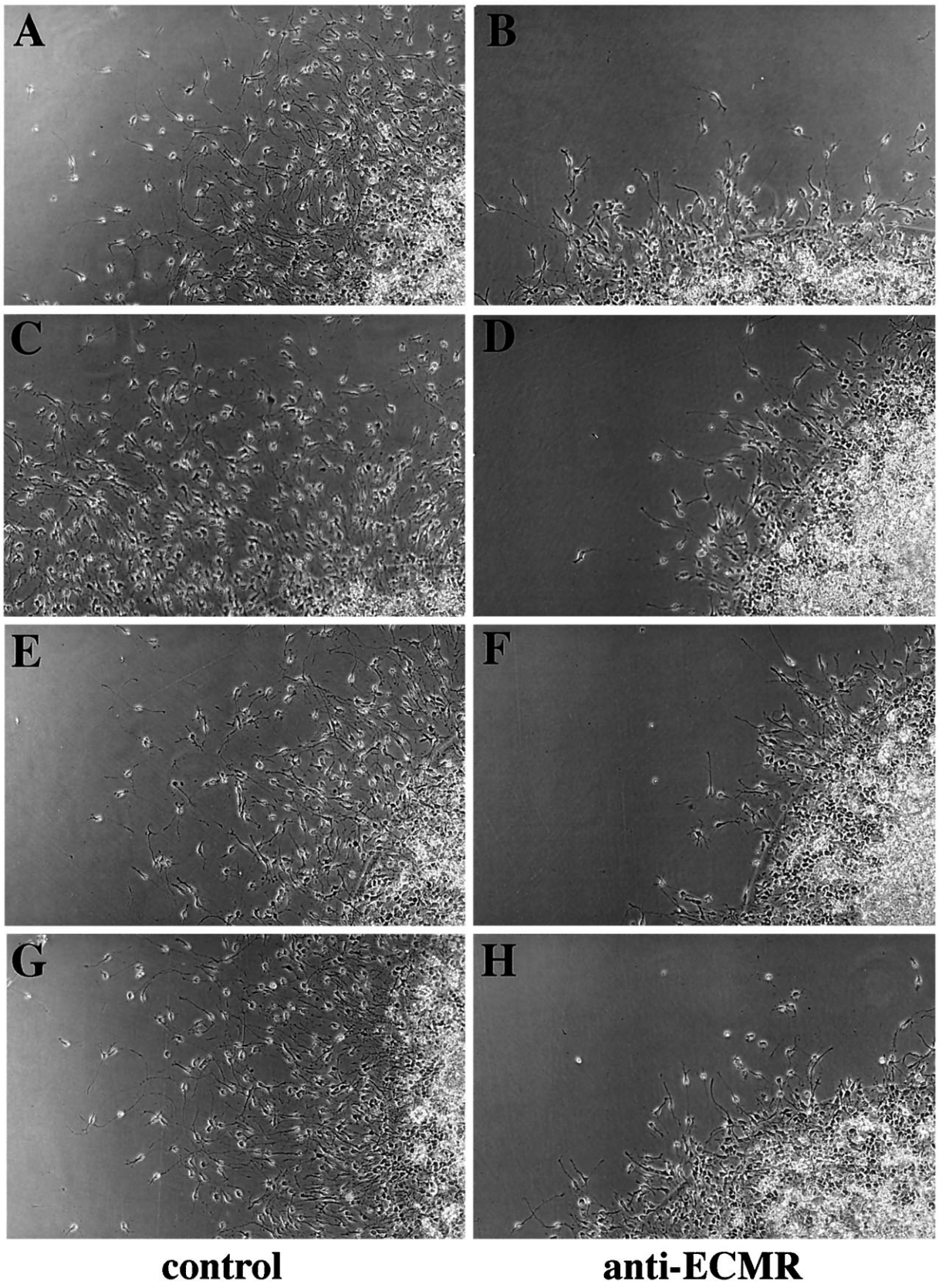

Figure 5. Effect of the anti-ECMR antiserum on oligodendrocyte precursor migration on different substrates. Cell migration away from agarose drops (prepared as described in Materials and Methods) after 2 d on poly-DL-ornithine (*A, B*), laminin (*C, D*), fibronectin (*E, F*), or vitronectin (*G, H*) is shown in the presence of either normal goat serum (*A, C, E, G*) or anti-ECMR antiserum (*B, D, F, H*), both at 1:400. Note that the anti-ECMR antiserum reduces oligodendrocyte precursor migration on all substrates tested but does not alter the bipolar morphology of the cells.

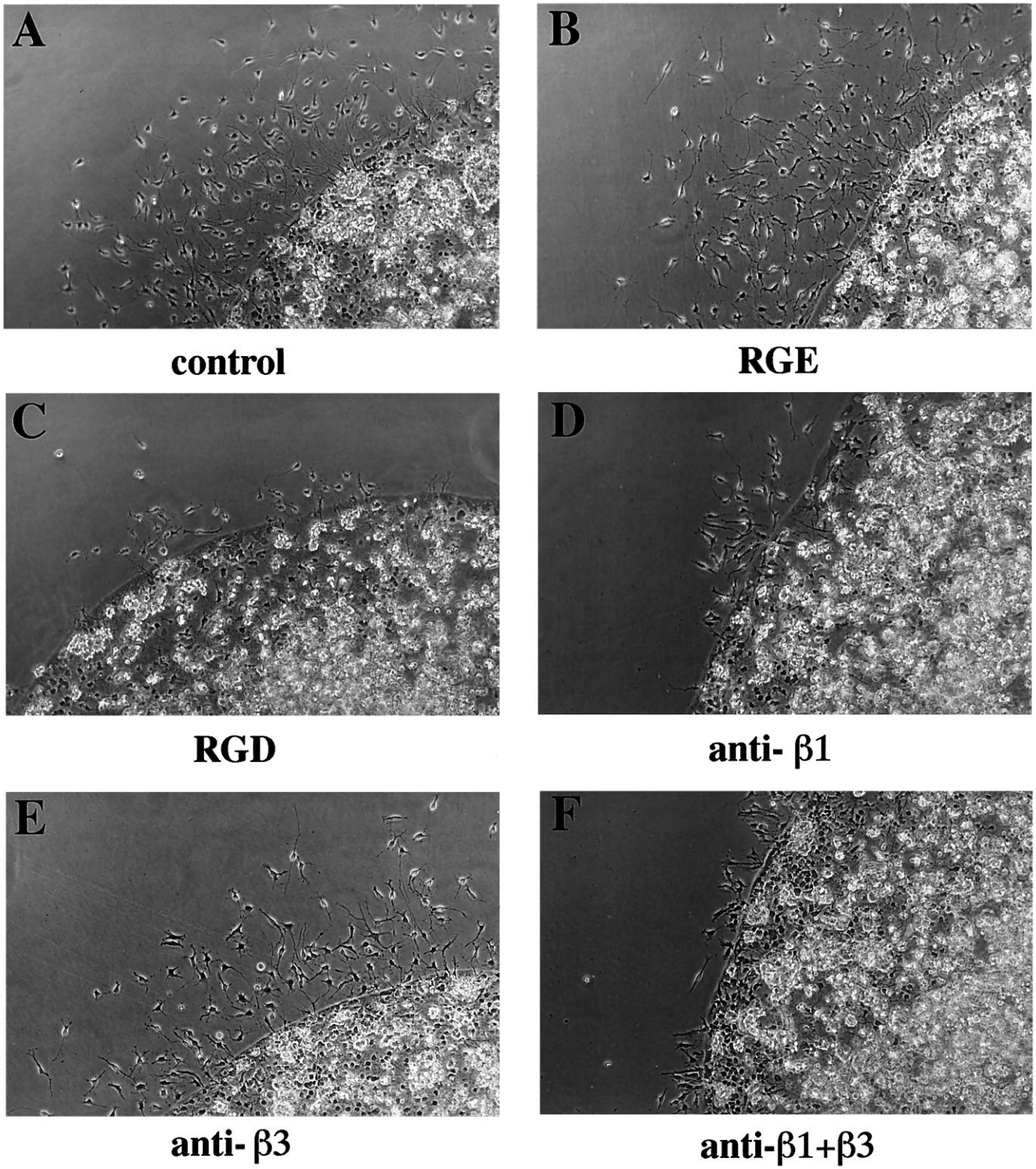

Figure 6. The effect of integrin inhibitors on oligodendrocyte precursor migration on AGM. Cell migration away from agarose drops (prepared as described in Materials and Methods) after 2 d on AGM is shown in the presence of normal rabbit serum (*A*), RGE peptide (*B*), RGD peptide (*C*), anti-β1 antiserum (*D*), anti-$\alpha v\beta 3$ antiserum (*E*), or anti-β1 and anti-$\alpha v\beta 3$ antiserum (*F*). The peptides were present at 0.1 mg/ml. Note that cell migration was inhibited both by RGD peptides and anti-β1 antiserum without any change to the morphology of the cells.

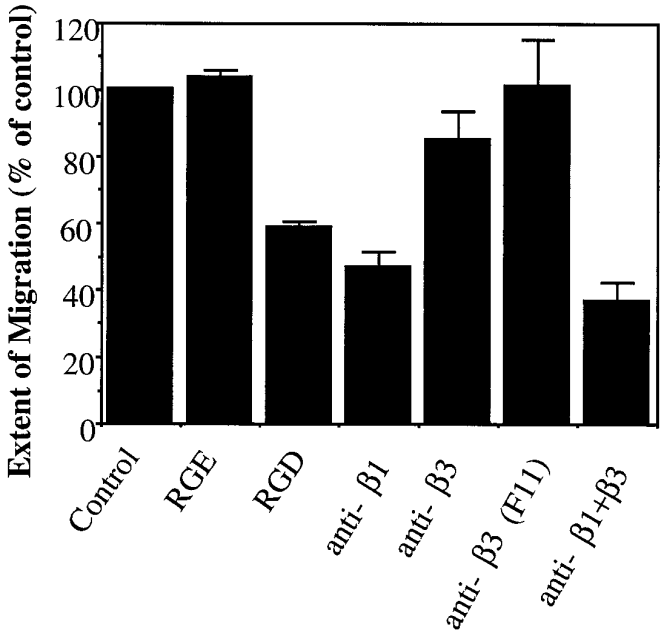

Figure 7. Quantification of the effect of integrin inhibitors on oligodendrocyte precursor migration on AGM. Cell migration away from agarose drops (prepared as described in Materials and Methods) after 2 d on AGM was measured in the presence of either normal rabbit serum, RGE peptide, RGD peptide, anti-β1 antiserum, anti-$\alpha v\beta 3$ antiserum, F11 (monoclonal anti-β3), or anti-β1 and anti-$\alpha v\beta 3$ antiserum in combination. The peptides were present at 0.1 mg/ml. Each point represents the mean ± SEM of three separate experiments. Note that cell migration was inhibited only by RGD peptides and anti-β1 antiserum.

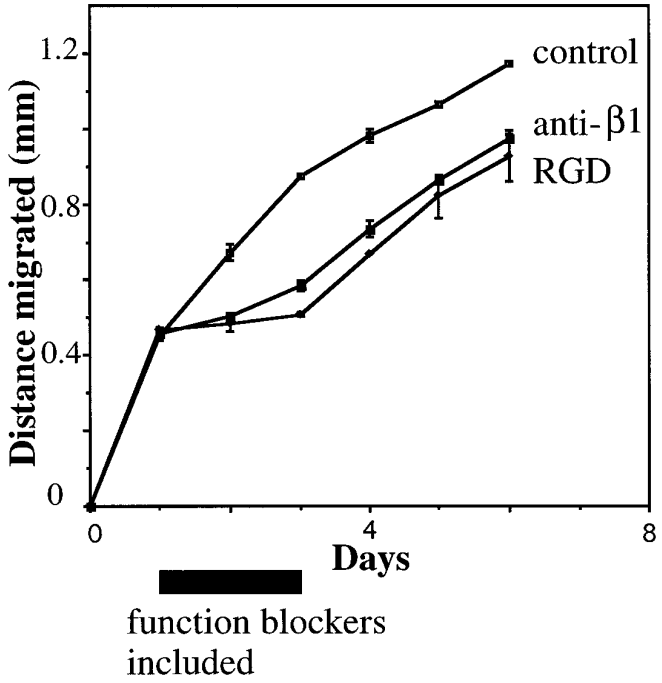

Figure 8. Migration of oligodendrocyte precursor cells after removal of integrin blockade. Cells were allowed to migrate away from agarose drops (prepared as described in Materials and Methods) for 1 d before the following reagents were introduced: RGE peptide (control), RGD peptide, or anti-β1 antiserum. After incubation for 2 d, the reagents were removed and cell migration was measured for an additional 3 d. Note that cells whose migration was blocked with either the RGD peptide or anti-β1 antiserum migrate at equal rates to the control cells once the blockade is removed.

is blocked with either RGD peptides or β1 antiserum migrate at a rate equal to the control cells once the block has been removed, showing them to be at equivalent stages of differentiation.

The $\alpha v\beta 1$ but not the $\alpha 6\beta 1$ integrin is involved in oligodendrocyte precursor migration on AGM

The preceding experiments show that oligodendrocyte precursor migration on AGM is blocked by both RGD peptides and anti-β1 integrin antibodies. Oligodendrocyte precursors express two different β1 integrins, $\alpha 6\beta 1$ and $\alpha v\beta 1$, either of which could be playing a role in precursor migration. Because the $\alpha 6\beta 1$ integrin is not RGD-dependent (Hall et al., 1990), but all αv integrins presently characterized are RGD-dependent (Koivunen et al., 1993), the inhibition of cell migration by RGD peptides would favor a role for $\alpha v\beta 1$ rather than $\alpha 6\beta 1$ in the migratory process. We addressed this question directly by using blocking antibodies directed specifically against $\alpha 6\beta 1$ or $\alpha v\beta 1$ on oligodendrocyte precursors, GoH3 and 9EG7, respectively. To perform these experiments, it was necessary to use mouse rather than rat oligodendrocyte precursor cells because of the species specificity of these antibodies. GoH3 is a well characterized function-blocking anti-α6 monoclonal antibody (Sonnenberg et al., 1987) that we have used previously to immunoprecipitate $\alpha 6\beta 1$ from mouse oligodendrocytes and their precursors (Milner and ffrench-Constant, 1994). Before the migration experiments, we performed adhesion assays confirming that GoH3 inhibited the function of $\alpha 6\beta 1$ on mouse oligodendrocyte precursors. This showed that GoH3 inhibited cell adhesion to laminin in a dose-dependent manner (0–10 μg/ml) but had no effect on adhesion to fibronectin (not shown). In addition, longer-term assays in which blocking antibodies were introduced after oligodendrocyte precursors had been allowed to attach for 30 min showed that GoH3 inhibited process extension during a 3 hr time course on laminin but not fibronectin (R. Milner and C. ffrench-Constant, unpublished observations). 9EG7 is a monoclonal antibody directed against the β1 subunit and has been shown to recognize this subunit only in certain conformations thought to be related to activation state. As a result, it can be classified as a "reporter" antibody (Lenter et al., 1993). Two lines of evidence show that 9EG7 recognizes $\alpha v\beta 1$ but not $\alpha 6\beta 1$ on oligodendrocyte precursors. First, as shown in Figure 9, 9EG7 immunoprecipitates the β1 subunit (running at 110 kDa) in association with an α subunit whose molecular weight corresponds to the dominant lower form of the αv subunit but not the α6 subunit immunoprecipitated by GoH3. We consistently see two αv subunits in these immunoprecipitation experiments on mouse but not rat oligodendroglia, as has also been described in *Pleurodeles* cells (Alfandari et al., 1995). Second, when used as a blocking antibody in oligodendrocyte precursor adhesion assays, 9EG7 significantly reduced the extent of sprouting on fibronectin (a recognized ligand for $\alpha v\beta 1$) but not on laminin (the $\alpha 6\beta 1$ ligand) (Fig. 10). We conclude from this that 9EG7 specifically recognizes the $\alpha v\beta 1$ integrin on oligodendrocyte precursors.

Migration assays with mouse oligodendrocyte precursor cells were carried out on the AGM substrate. Unlike the experiments with rat cells, which were performed in serum-free conditions, the experiments with mouse cells were carried out in the presence of 1% horse serum to promote survival of mouse oligodendrocyte precursor cells. As shown in Figure 11, the anti-β1 antiserum inhibited the migration of mouse oligodendrocyte precursor cells on AGM, as it had on rat, reducing migration after 2 d to 33.13 ±

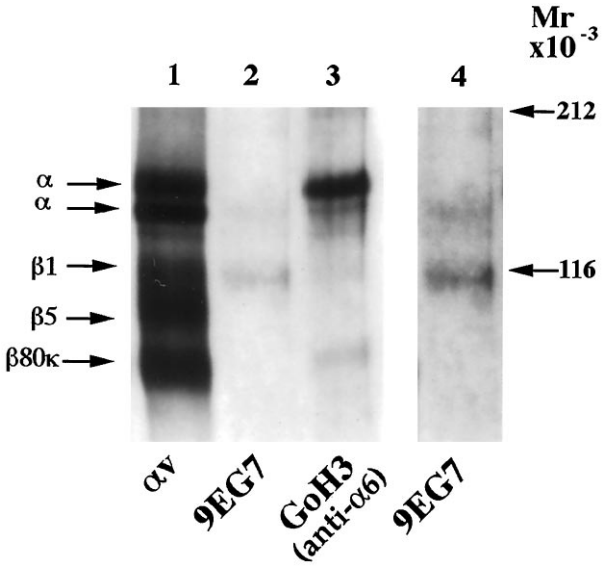

Figure 9. Specificity of the 9EG7 monoclonal antibody for oligodendroglial $\alpha v\beta 1$ integrin. Immunoprecipitations of biotin-labeled cell-surface proteins from mouse oligodendrocyte precursors were performed as described in Materials and Methods, with either anti-αv antiserum (*lane 1*), the 9EG7 monoclonal antibody (*lane 2*), or the anti-$\alpha 6$-specific GoH3 monoclonal antibody (*lane 3*). The proteins were separated on a nonreducing gel. *Lanes 1–3* represent three adjacent lanes on the gel that have been exposed for equal lengths of time, whereas *lane 4* represents a longer exposure of *lane 2*. Note that the 9EG7 antibody immunoprecipitates the $\beta 1$ subunit associated with an α subunit that comigrates with the dominant αv subunit but not the $\alpha 6$ subunit.

10.4% of the control ($p < 0.01$). The anti-$\alpha v\beta 1$ monoclonal antibody 9EG7 also inhibited migration, reducing migration to 58.4 ± 17.6% of the control ($p < 0.05$). In contrast, the anti-$\alpha 6$ blocking antibody GoH3 did not reduce the extent of migration. As before, no changes in morphology were seen in association with these changes in migration, although the mouse oligodendrocyte precursors grown under these conditions do not show the characteristic bipolar morphology seen in the rat cells, being rather less polarized and with a greater number of short processes (Fig. 11).

$\alpha v\beta 1$ but not $\alpha 6\beta 1$ promotes oligodendrocyte precursor migration on a laminin/fibronectin/vitronectin composite substrate

The results with the anti-$\alpha v\beta 1$ 9EG7 and anti-$\alpha 6$ GoH3 monoclonal antibodies suggest that the $\alpha v\beta 1$ but not the $\alpha 6\beta 1$ integrin plays the dominant role in promoting oligodendrocyte precursor migration on AGM. This result might be expected, however, if the $\alpha 6\beta 1$ ligand laminin was either masked or not present in the AGM. To address this issue, experiments were carried out both on laminin substrates (which promote adhesion, outgrowth, and migration, as described above) and on a composite ECM substrate coated with a solution containing 10 μg/ml of laminin, fibronectin, and vitronectin. As shown in Figure 12, the anti-$\alpha 6$ monoclonal antibody GoH3 did not significantly reduce oligodendrocyte precursor migration on either laminin substrates or the composite matrix of fibronectin, laminin, and vitronectin ($p > 0.05$). In contrast, anti-$\beta 1$ antiserum reduced the extent of migration on the composite substrate to 46.03 ± 1.98% of the control ($p < 0.001$). This result shows that $\alpha 6\beta 1$ is not playing a significant role in oligodendrocyte precursor migration, even when the $\alpha 6\beta 1$ ligand laminin is present within the substrate.

DISCUSSION

In this study we have used the Varani migration assay to investigate the role of ECM/integrin interactions in oligodendrocyte precursor migration. Two major conclusions have emerged. First, the purified ECM substrates laminin, fibronectin, and vitronectin, as well as an ECM secreted by astrocytes, are effective at promoting cell migration, whereas collagen is not. Second, oligodendrocyte precursor migration on AGM is inhibited by blocking $\alpha v\beta 1$ but not $\alpha 6\beta 1$, suggesting a dominant role for the $\alpha v\beta 1$ integrin in the migration process.

The agarose drop assay as a method of investigating oligodendrocyte precursor migration on ECM substrates

The mechanisms regulating the migration of oligodendrocyte precursor cells has been addressed by *in vitro* studies using both time-lapse microscopy (Small et al., 1987; Noble et al., 1988; Kiernan and ffrench-Constant, 1993) and chemotactic chamber assays (Armstrong et al., 1990; Frost et al., 1996). In the current study we have used a novel method based on the Varani agarose drop assay. In the presence of PDGF, oligodendrocyte precursor cells at the periphery of the drop start to migrate away within hours and continue their migration for at least 7 d. Unlike time lapse, this method permits the simultaneous analysis of different experimental conditions. It also has advantages over the use of the chemotaxis chamber, including the ability to monitor cell migration at intermediate time points, to change experimental conditions within an experiment, and to examine cell morphology during the experiment. A potential problem is that these experiments continue for several days, and the extent of cell dispersal from the drop will reflect both cell migration and proliferation. Time-lapse analysis, however, shows that there is consistent migration of precursors at all time points examined. Furthermore, migration of precursors still occurs in the presence of the mitotic inhibitor aphidicholin, showing that cell division is not a major factor in the extent of dispersal in the assay.

Involvement of ECM and integrins in oligodendrocyte precursor migration

The ECM molecules laminin, fibronectin, and vitronectin, and a complex ECM mixture derived from astrocytes (AGM) all promote cell migration. These results are in agreement with parallel experiments performed in our laboratory showing that both fibronectin and merosin, a member of the laminin family, promote oligodendrocyte precursor migration within a modified chemotaxis chamber assay (Frost et al., 1996). Our findings also show that integrins are involved in oligodendrocyte precursor migration on these ECM substrates. The anti-ECMR antibody, which recognizes all oligodendroglial integrins, blocked cell migration. Having shown previously that oligodendroglial cells express $\alpha 6\beta 1$ and several αv integrins, we targeted these specific integrins with RGD peptides and function-blocking antibodies. The RGD peptides and anti-$\beta 1$ antiserum both reduced migration on AGM to ~50% of the control value. As argued in the results, the RGD effect pointed to the involvement of $\alpha v\beta 1$ integrin in migration. This was confirmed directly by using two monoclonal antibodies: G0H3, specific to $\alpha 6\beta 1$ (Sonnenberg et al., 1987), and 9EG7, which we find is specific for the $\alpha v\beta 1$ integrin expressed by oligodendrocyte precursors, despite the fact that they also express other $\beta 1$ integrins. The specificity of this antibody for a subset of expressed $\beta 1$ integrins was shown on lymphocytes in the original paper describing 9EG7 (Lenter et al., 1993), and we have also

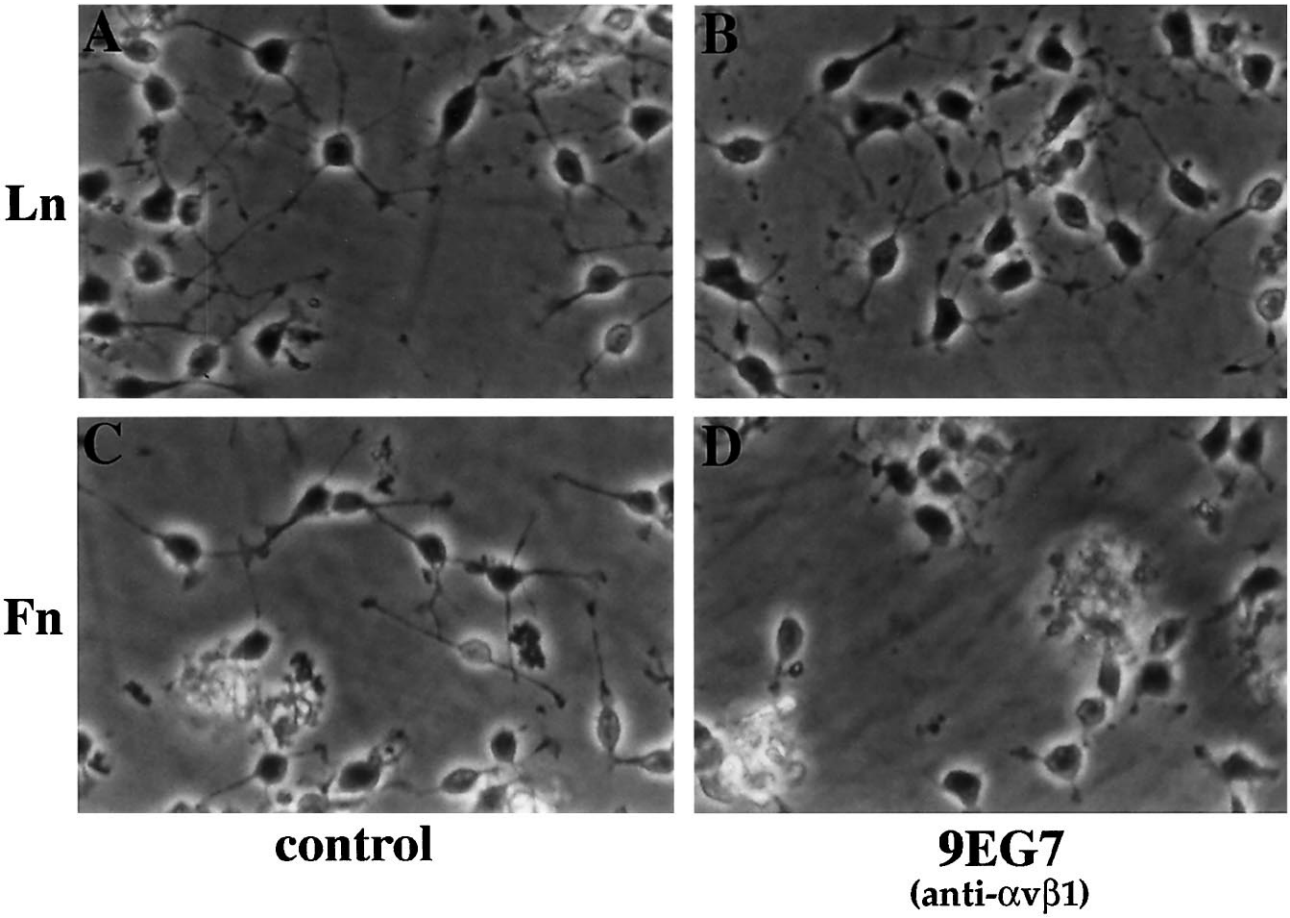

Figure 10. Specific function-blocking effect of the anti-$\alpha v\beta 1$ 9EG7 monoclonal antibody. Mouse oligodendrocyte precursors were plated onto either laminin (*A, B*) or fibronectin (*C, D*) in the absence (*A, C*) or presence (*B, D*) of the 9EG7 monoclonal antibody, and allowed to adhere and process for 1 hr. Note that 9EG7 inhibits process outgrowth on fibronectin but not laminin.

observed this selectivity on fibroblasts, where 9EG7 immunoprecipitated only $\alpha 1\beta 1$, despite the expression of higher levels of $\alpha 5\beta 1$ (R. Milner and C. ffrench-Constant, unpublished observations). As such, 9EG7 represents a reporter antibody that recognizes a subset of the $\beta 1$ integrins expressed on different cell types, presumably reflecting different conformations of these integrins relating to activation state (Lenter et al., 1993).

The observation that $\alpha 6\beta 1$ seems to play only a limited role in migration on AGM raises the question as to why the $\alpha 6\beta 1$ ligand laminin is so effective at promoting precursor migration. One possibility is that the uncharacterized $\alpha v\beta 80$k integrin expressed by oligodendrocyte precursors (Milner and ffrench-Constant, 1994) may bind laminin and would not be blocked by GoH3. Alternatively, oligodendrocyte precursor cells may also express nonintegrin laminin receptors.

An interesting conclusion from our results is that blocking oligodendrocyte precursor migration is not associated with any changes in differentiation. This follows from the observation that precursors maintain the bipolar morphology of undifferentiated cells during migration blockade. Moreover, precursor cells released from migration blockade resume migration at the same speed as control cells. Taken together, these experiments show that the signaling pathways regulating migration and differentiation can be separated. This is in contrast to the situation with proliferation and differentiation, because cells cultured in the absence of mitogenic factors differentiate constitutively into oligodendrocytes once they drop out of division (Temple and Raff, 1985, 1986).

A role for the $\alpha v\beta 1$ integrin in cell migration

Our finding that $\alpha v\beta 1$ is a migration-promoting integrin for oligodendrocyte precursors is consistent with previous studies showing that $\beta 1$ integrins promote migration in other cell types. This has been shown in the neural crest cell lineage using $\beta 1$ integrin function-blocking antibodies (Bronner-Fraser, 1986) and in neuronal precursors of the developing tectum using antisense cDNA to reduce levels of $\beta 1$ (Galileo et al., 1992). More recently, the $\beta 1$ integrin gene has been knocked out in F9 teratocarcinoma cells and in embryonic stem (ES) cells. The ES cells were then unable to migrate toward a chemotactic source in Boyden chambers (Fassler et al., 1995), whereas $\beta 1$-deficient F9 teratocarcinoma cells lost their ability to migrate on fibronectin and vitronectin substrates, despite expressing at least two other vitronectin receptors, $\alpha v\beta 3$ and $\alpha v\beta 5$ (Stephens et al., 1993). As in the F9 cells, our data indicate that $\alpha v\beta 3$ is not playing a major role in promoting oligodendrocyte precursor cell migration. This is in contrast to other studies in different cell types supporting a role for $\alpha v\beta 3$ in migration both *in vitro* (Leavesley et al., 1992; Delannet et al., 1994) and during metastatic invasion *in vivo* (Albelda et al., 1990; Gehlsen et al., 1992; Seftor et al., 1992). To our knowledge, the present study provides the first evidence for a role of $\alpha v\beta 1$ in migration.

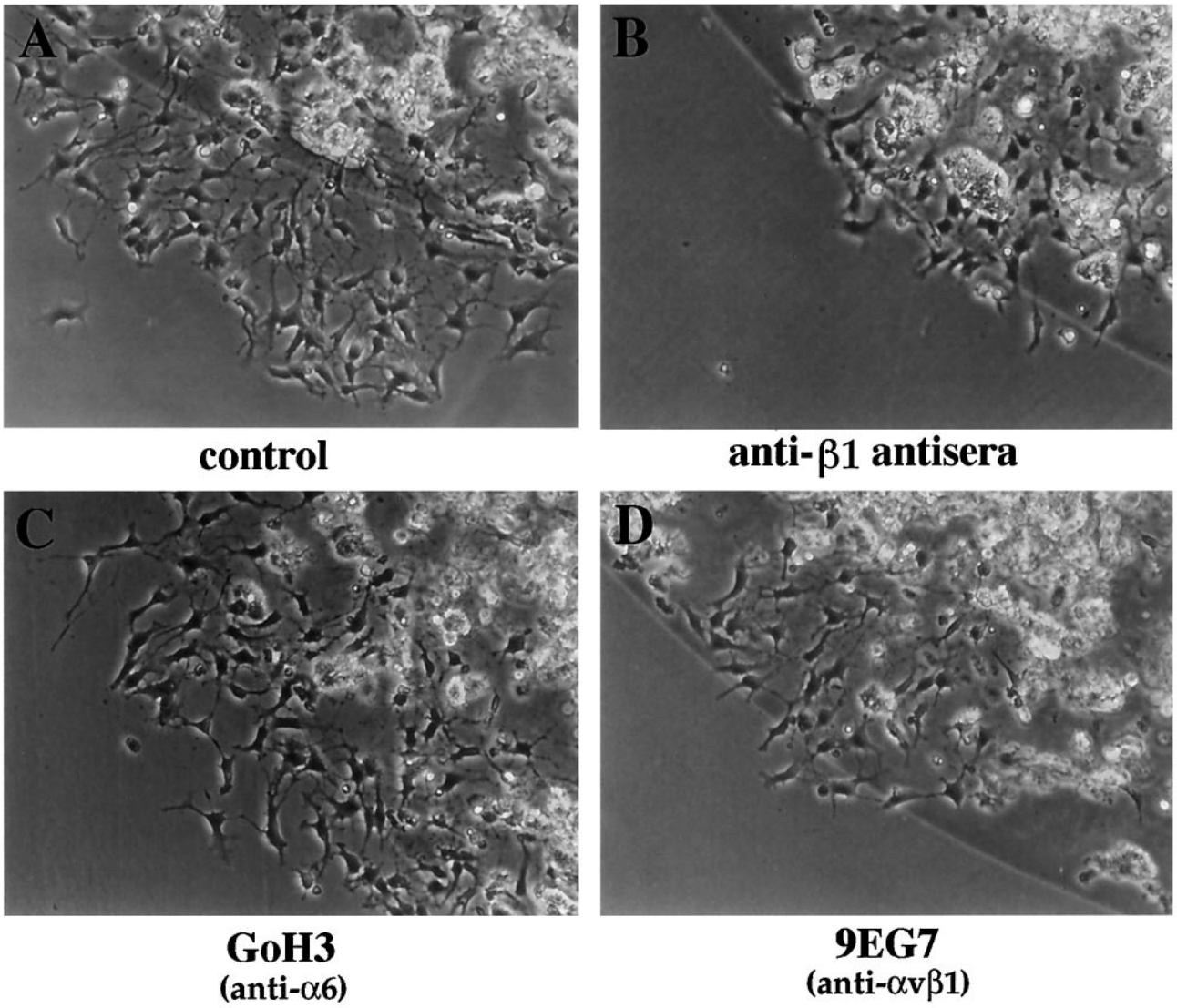

Figure 11. The effect of integrin inhibitors on oligodendrocyte precursor migration on AGM. Mouse oligodendrocyte precursor cell migration away from agarose drops (prepared as described in Materials and Methods) after 2 d on AGM was measured under control conditions (*A*) or in the presence of anti-β1 antiserum (*B*), the anti-α6 GoH3 monoclonal antibody (*C*), or the anti-αvβ1 9EG7 monoclonal antibody (*D*). Note that cell migration was inhibited by the anti-β1 antiserum and the anti-αvβ1 9EG7 monoclonal antibody but not by the anti-α6 GoH3 antibody.

This work raises the question as to the nature of the αvβ1 ligand *in vivo*. Several ligands have been described, including fibronectin (Vogel et al., 1990), vitronectin (Bodary and McLean, 1990), and fibrinogen and osteopontin (Liaw et al., 1995). Both fibronectin and vitronectin are present within white matter tracts early in development (Chun and Shatz., 1988; Neugebauer et al., 1991; Sheppard et al., 1991). Although the precise distribution of fibronectin during the process of myelination has not yet been established, recent evidence shows vitronectin to be a candidate ligand for interaction with oligodendroglial integrins during myelination. Immunocytochemical studies in adult rat brain show that vitronectin is expressed within several different myelinated tracts (Einheber et al., 1996). The presence of a known ECM ligand for αvβ1 in myelinated tracts suggests that interactions between oligodendroglial integrins and ECM will occur *in vivo*, although immunocytochemical studies in earlier developmental stages are required. Based on our cell culture data, it is likely that any such interactions will play important instructive roles in regulating oligodendroglial migration.

Two other possibilities exist for the αvβ1 ligand. First, Cardwell and Rome (1988a) identified an RGD-blockable component within AGM that was not recognized by antibodies against fibronectin or vitronectin, raising the possibility that a novel αvβ1 ligand might be secreted by astrocytes. Second, the ligand *in vivo* may be a cell-surface molecule rather than an ECM molecule. Other β1 and αv integrins recognize cell surface ligands: α4β1 binds VCAM (Elices et al., 1990), whereas α6β1 binds fertilin (Almeida et al., 1995), a member of the ADAM family of cell-surface molecules (Wolfsberg et al., 1995). More recently, both α5β1 and αvβ3 have been shown to interact with L1 (Ruppert et al., 1995; Montgomery et al., 1996). It is possible, therefore, that αvβ1 binds an unidentified cell-surface molecule within axonal tracts, such as L1, and that this provides a mechanism for guidance of migrating oligodendroglia.

These observations complement previous work showing a role for growth factors in the control of oligodendrocyte precursor migration (Small et al., 1987; Noble et al., 1988; Armstrong et al., 1990; Kiernan and ffrench-Constant, 1993; Frost et al., 1996).

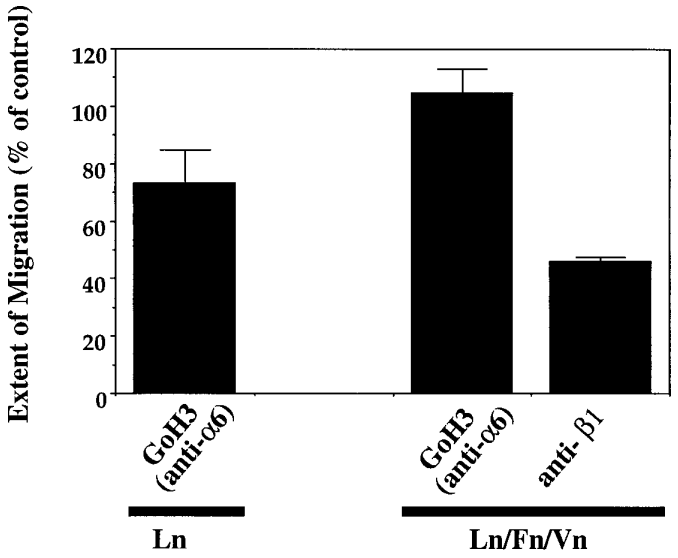

Figure 12. The effect of $\alpha v\beta 1$ and $\alpha 6\beta 1$ inhibition on oligodendrocyte precursor migration over ECM substrates. The extent of mouse oligodendrocyte precursor cell migration away from agarose drops (prepared as described in Materials and Methods) after 2 d on either laminin or a composite laminin/fibronectin/vitronectin substrate was measured in the presence of the anti-$\alpha 6$ GoH3 monoclonal antibody or anti-$\beta 1$ antiserum. The extent of migration is presented as a percentage of migration observed under control conditions, with no antibody present. Note that the anti-$\alpha 6$ GoH3 antibody had no significant inhibitory effect on either laminin or the composite substrate ($p > 0.05$), whereas the anti-$\beta 1$ antiserum significantly inhibited migration on the composite substrate ($p < 0.001$).

They emphasize that the control of migration *in vivo* will be regulated by both integrin- and growth factor-mediated signaling pathways and as such add significantly to our understanding of oligodendrocyte precursor cell biology. Our results also suggest a model by which integrins might regulate oligodendrocyte precursor migration. Oligodendroglia lose migratory potential on differentiation, and in this study we have shown that this occurs over 7 d. During the same time scale, there is a loss of $\alpha v\beta 1$ from the cell surface and expression of $\alpha v\beta 5$ (Milner and ffrench-Constant, 1994). Given that the $\alpha v\beta 1$ integrin promotes migration, the developmental switch of αv-associated β subunits may play a key role in regulating the timing of oligodendroglial migration. A prediction of this model is that overexpression of the $\alpha v\beta 1$ heterodimer may enhance the migration of oligodendroglial cells *in vivo*. If so, this might have useful implications for future therapeutic strategies using transplanted oligodendrocyte precursors to repair widespread demyelinated lesions.

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