Neural Progenitor Cells Lack Immunogenicity and Resist Destruction as Allografts

JUNKO HORI,a,b TAT FONG NG,a MARIE SHATOS,a HENRY KLASSEN,c J. WAYNE STREILEIN,c MICHAEL J. YOUNG,a

aDepartment of Ophthalmology, Schepens Eye Research Institute, Harvard Medical School, Boston, Massachusetts, USA; bDepartment of Ophthalmology, Nippon Medical School, Tokyo, Japan; cStem Cell Research, Children’s Hospital of Orange County, Orange, California, USA

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ABSTRACT

Multipotent, self-renewing stem and progenitor cells isolated from the mammalian central nervous system (CNS) have been shown to survive as allografts following transplantation to sites throughout the neuraxis. However, studies of this type shed little light upon the immunologic properties of the cells themselves, primarily because little is learned about the intrinsic immunogenic properties of a cell when it is grafted into an immune-privileged site. We have therefore investigated the immunogenic and antigenic properties of CNS progenitor cells by grafting them into a conventional (i.e., non-immune-privileged) site, namely, beneath the kidney capsule. Our results indicate that allogeneic CNS progenitor cells survive at least 4 weeks in a conventional site, during which time they neither sensitize their hosts nor express detectable levels of major histocompatibility complex (MHC) class I or II. These in vivo data are in accord with flow cytometric results showing that CNS progenitor cells do not express MHC class I or class II, either at baseline or upon differentiation in 10% serum. Exposure to interferon gamma, however, reversibly upregulates expression of these key transplantation antigens. Together, these results reveal CNS progenitor cells to possess inherent immune privilege. Since CNS progenitor cell allografts were rejected beneath the kidney capsule following specific sensitization of the host, CNS progenitor cells were able to display alloantigens, albeit not in an immunogenic form.

INTRODUCTION

Immune privilege was discovered by Medawar in the late 1940s during the course of his classic studies of the immunobiology of tissue transplantation. Medawar’s studies revealed the existence of several so-called immune-privileged sites, including the brain and the anterior chamber of the eye [1]. Later, investigators demonstrated that immune privilege could also be a property of particular organs and tissues. The cornea [2] and the testis [3] are well-studied examples of immune-privileged tissues. Over the past 20 years or so, investigators and clinicians have made attempts to transplant functional brain and retinal...
tissues in an effort to alleviate a variety of central nervous system (CNS) and retinal disorders. Rather little success has attended these experiments, prompting a reevaluation of two important questions: First, does immune privilege actually exist within the brain and the eye? Second, are brain and retinal tissues immune privileged? Renewed immunologic studies of the brain and the eye during the past 20 years have reaffirmed the existence of immune privilege within the brain [4] and within three major intraocular compartments, namely, the anterior chamber, the vitreous cavity [5], and the sub-retinal space [6]. However, the question of the immune-privileged status of brain and retinal tissue is unresolved, primarily because virtually all studies have placed these neuroectodermally derived tissues into established immune-privileged sites. It is impossible to discern the extent to which a particular tissue is immune privileged if grafts of that tissue are implanted in sites that are themselves immune privileged. Instead, assessment of a tissue as immune privileged requires that the tissue be grafted to a nonprivileged site where its vulnerability to immune rejection is not limited by the site itself.

Recently, a new cell type known as neural stem cell or neural progenitor cell has been isolated from various regions of the adult and embryonic CNS of mice, rats, and humans, among other species [7]. These cells are unique in that they are multipotent, i.e., they can give rise to the three cell lineages of the CNS: neurons, astrocytes, and oligodendrocytes. Moreover, neural stem cells are self-renewing, i.e., they divide to give rise to at least one daughter cell that maintains multipotency and self-renewal, leaving the other daughter cells free to follow a differentiation pathway. The inherent plasticity of neural stem cells has generated interest in whether these cells can be used to replace cells in the mammalian CNS. Indeed, studies in animal models have shown that neural stem cells can specifically replace populations of diseased or damaged cells, in some cases leading to behavioral recovery [8]. The fact that these cells can be grown in large numbers ex vivo represents another advantage over conventional solid tissue grafts, particularly with respect to potential clinical applications.

We have recently reported that neural stem cells derived from rat hippocampus were able to integrate into degenerating retinas of both rd mice and RCS rats [9]. No evidence of immune rejection was discerned in the experiments, thereby raising further questions concerning the immunogenicity of neural stem or progenitor cells. In particular, might neural progenitor cells function as an immune-privileged tissue?

To examine this issue, we implanted syngeneic (transgenic GFP-C57BL/6 to C57BL/6) and allogeneic (transgenic GFP-C57BL/6 to BALB/c) neural progenitor cells beneath the kidney capsule of adult mice [10]. As these mouse strains are inbred, syngeneic grafts should be accepted as if they were autografts (i.e., survive indefinitely), while allografts between defined strains of mice should give rise to a consistent and reproducible immune response.

These cells are described as “progenitor” rather than “stem” cells because they were not clonally derived (instead passaged in medium- to high-density culture) and were transplanted as spheres, which in fact consist of a subpopulation of true stem cells and a larger population of more committed progenitor cells. These implants were evaluated for survival by clinical inspection and postmortem immunohistochemical analysis performed at periodic intervals postimplantation. The ability of allogeneic neural progenitor cells to sensitize recipients when implanted beneath the kidney capsule was assessed. We also evaluated the vulnerability of implanted cells to rejection following specific sensitization of the recipient to transplantation antigens of the graft donor. Our results strongly suggest that neural progenitor cells possess the highly desirable characteristic of being immune-privileged cells, thus enhancing their potential utility in the setting of CNS repair.

**Materials and Methods**

**Mice and Anesthesia**

Male BALB/c (H-2d) and C57BL/6 (H-2b) mice were purchased from Taconic Farm (Germantown, NY; http://www.taconic.com). Male enhanced green fluorescent protein (EGFP) transgenic mice [11] (C57BL/6 background, kindly provided by Dr. Okabe, University of Osaka) were bred in our animal colony. All mice were 8-10 weeks of age and treated according to the Association for Research in Vision and Ophthalmology Resolution on the use of animals in research. Each mouse was anesthetized by i.m. injection with a mixture of 3.75 mg ketamine and 0.75 mg xylazine before all surgical procedures.

**Isolation of CNS Progenitor Cells from EGFP Transgenic Mice**

CNS progenitor cells were isolated from the whole brain of postnatal day 1 GFP transgenic C57BL/6 mice [11] and maintained using methods previously described [12]. Briefly, whole brains were surgically removed from GFP transgenic C57BL/6 mice at postnatal day 1 and immediately placed into phosphate-buffered saline (PBS) containing 300 μg/ml penicillin-streptomycin. Tissue was minced into 1-2-mm³ pieces and digested with 0.1% Type 1 collagenase for 20 minutes. The supernatant containing liberated cells was collected and forced through a 100-μm mesh sievel. The cells were centrifuged and seeded in Dulbecco’s modified Eagle’s medium (DMEM)/Hams F-12 medium high glucose supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and
Neonatal Cerebellar Tissue Isolation

As a control for passaged neural progenitor cell grafts, we also isolated uncultured or passaged neural tissue from the brains of postnatal day 1 GFP transgenic C57BL/6 mice. Animals were euthanized, and whole brains were removed and placed into Hank’s balanced salt solution (HBSS). The meninges were dissected, and the cerebellum was isolated. The cerebellum was then minced with two #10 scalpels blades until small pieces the size of the progenitor grafts (500-1,000 µm in diameter) were obtained. These were then grafted as described for the CNS progenitor cells below.

Differentiation and Characterization of CNS Progenitor Cells

In Vitro

To examine the differentiation of GFP-expressing CNS progenitor cells in vitro, several spheres were plated into 24-well dishes in DMEM/F12 medium supplemented with 10% FBS. Cultures were fixed with 4% paraformaldehyde at 3 days, 1 week, 2 weeks, and 4 weeks after plating. Cultures were blocked in 1% bovine serum albumin (BSA) + 0.2% Triton X-100 or 1% BSA + 10% normal goat serum for 30 minutes. Cultures were then incubated with primary antibody for Ki-67 (1:100; Vector Labs; Burlingame, CA; http://www.vectorlabs.com), nestin (1:1; DSHB, University of Iowa; http://www.uiowa.edu/~dshbwww), NF200 (1:1000; Sigma; St. Louis, MO; http://www.sigmaaldrich.com), glial fibrillary acidic protein (GFAP; 1:50; Zymed Laboratories; South San Francisco, CA; http://www.zymed.com), and GalC (1:50; Sigma) for 1 hour. After rinsing in PBS, cultures were followed by incubation in species-specific IgG conjugated to Cy3 for 1 hour (1:100; Jackson ImmunoResearch Labs; West Grove, PA; http://www.jacksonimmuno.com).

Heterotopic Transplantation of Neural Progenitor Cells Under the Kidney Capsule

BALB/c, C57BL/6, or BALB/c anti-C57BL/6 mice were used as recipients of neural progenitor cells (or freshly isolated postnatal day 1 cerebellum) derived from the brain of EGFP transgenic mice. We implanted into syngeneic (transgenic GFP-C57BL/6 to C57BL/6), allogeneic (transgenic GFP-C57BL/6 to BALB/c), and allogeneic primed (transgenic GFP-C57BL/6 to BALB/c anti-C57BL/6) adult mice. Each experimental panel was composed of at least 10 recipients. A cluster of neurospheres approximately 500-1,000 µm in diameter (containing approximately 500,000 cells) was placed beneath the kidney capsule using established techniques [13]. A similar sized cluster of freshly isolated brain tissue was grafted in control animals. To place the grafts at the heterotopic site, a skin incision was made in the left flank of recipient mice, and the muscle wall was incised and the kidney exteriorized. A subcapsular pocket was created between the kidney and the kidney cortex, and the graft was placed into the pocket. The kidney was replaced in the abdominal cavity and the skin was closed with 7-mm clips.

In Vivo Evaluation of Heterotopic Neural Progenitor Cell Grafts

Heterotopic neural progenitor cell graft survival was assessed by visual inspection under the operating microscope at selected times postimplantation. At each time point, graft-bearing mice were anesthetized, the kidney was exteriorized, and in vivo assessment was performed through a binocular surgical microscope, including evaluation of graft appearance and vascular ingrowth.

Immunochemistry and Characterization of Heterotopic Neural Progenitor Cells

Immunochemical studies for CD45, H-2Kb, and I-Ab expression were performed on frozen sections (7 µm) of grafts placed under the kidney capsule. Phycoerythrin (PE)-labeled rat anti-mouse CD45, PE-labeled rat anti-mouse H-2Kb, and PE-labeled rat anti-mouse I-Ab monoclonal antibodies (Pharmingen; San Diego, CA; http://www.bdbiosciences.com/pharmingen) were used as primary antibodies. Graft-bearing kidneys were removed at 1 hour, day 7, day 14, and day 28, and were fixed in 4% paraformaldehyde. Most of the kidney tissue was cut away, leaving the neural progenitor cell graft in place. For confocal microscopy, the small piece of graft-bearing kidney was incubated in PE-anti-CD45, PE-anti-H-2Kb, and PE-anti-I-Ab antibodies diluted to 4 µg/ml for 2 hours at room temperature. After washing with PBS, the samples were mounted on slides and observed by confocal microscopy.
Delayed Hypersensitivity Assessment

At selected times after allogeneic neural progenitor cell implantation beneath the kidney capsule, delayed hypersensitivity was assessed by ear challenge of spleen cells from C57BL/6 mice. Spleens were excised from C57BL/6 mice (6–8 weeks) and were pressed through a Nylon cell strainer (70 µm, Falcon; Billings, MT) to prepare monocellular suspensions. Spleen cells were washed twice in red blood cell lysing buffer (Sigma) and resuspended in HBSS. All panels of test mice included 5-6 animals. As a positive control, a similar number of irradiated spleen cells were injected into the ear pinna of normal BALB/c mice, which were immunized 1 week previously by s.c. injection of 10 × 10⁶ C57BL/6 spleen cells into the contralateral ear. As a negative control, 1 × 10⁶ irradiated spleen cells were injected into ear pinna of naive mice. Twenty-four and 48 hours after ear injection, ear thickness was measured with a low-pressure engineer’s micrometer (Mitutoyo, MTI Corporation; Paramus, NJ; http://www.southerntool.net/frame_mitutoyo.html). Ear swelling was expressed as follows: specific swelling = ([24-hour numerical values of right ear – 0-hour numerical values of right ear] – [24-hour numerical values of left ear – 0-hour numerical values of left ear]) × 10⁻³ mm. Ear swelling responses at 24 hours after ear injection are presented as a group mean ± standard error (SE).

In order to evaluate the capacity of neural progenitor cells to present their alloantigens in a manner that could be recognized by sensitized T cells, panels of BALB/c mice were presensitized with C57BL/6 spleen cells injected into the ear pinna at day 25 posttransplantation. Seven days later (day 32), test mice received intra-pinna ear challenge (in the contralateral ear) with 1 × 10⁶ irradiated C57BL/6 neural progenitor cells, whereas positive-control mice received challenge with 1 × 10⁶ irradiated C57BL/6 spleen cells. Negative-control mice were only ear challenged with C57BL/6 spleen cells. Ear swelling was measured at 24 and 48 hours after injection. This experiment was repeated three times with similar results. Data from a representative experiment are displayed.

Flow Cytometry

Cultured CNS progenitor cells used for flow cytometry to assess baseline expression levels of surface molecules were grown in uncoated flasks under standard proliferation conditions, as described above. Additional CNS progenitor cells were grown for 8 days under differentiation conditions consisting of medium identical to the above but containing 10% sterile-filtered fetal calf serum (FCS) instead of EGF. Other cultures were grown under major histocompatibility complex (MHC)-induction conditions using proliferation medium supplemented with recombinant mouse interferon (IFN)-γ at 33 ng/ml (R&D Systems; Minneapolis, MN; http://www.rndsystems.com). After 4 days, remaining cells were returned to standard proliferation conditions by complete exchange of IFN-γ-containing medium for proliferation medium and maintained for an additional 11 days.

Cells for analysis were harvested using a trypsin/EDTA solution (Irvine Scientific; Santa Ana, CA; http://www.irvinesci.com) and resuspended in Ca²⁺/Mg²⁺-free PBS containing 1% human albumin and 0.02% sodium azide. Each sample was divided into aliquots containing approximately 5 × 10⁶ cells and monoclonal antibodies were added. After 20 minutes of incubation, cells were washed with PBS and a labeled secondary was added, followed by an additional 15 minutes of incubation in the dark and a final wash. Cells were then resuspended in PBS containing 7-amino Actinomycin D (1 µg/ml) and cytometry was performed on a FACS Vantage (BD Biosciences; San Diego, CA; http://www.bdbiosciences.com) equipped with a 488-nm argon laser.

Primary antibodies used for flow cytometry (BD Pharmingen) were unconjugated mouse IgG. Secondaries were PE-conjugated sheep anti-mouse IgG (Sigma) or streptavidin-PE (Immunotech; Miami, FL; http://www.beckmancoulter.com). Isotypes were from BD Pharmingen or Sigma.

Statistical Analyses

Ear swelling measurements were evaluated statistically using a two-tailed Student’s t-test. All probability values of p < 0.05 were deemed significant.

RESULTS

Differentiation and Characterization of CNS Progenitor Cells In Vitro

Following exposure to DMEM/F12 medium supplemented with 10% FBS, neural progenitor cell spheres formed adherent monolayer cultures. They followed a predictable pattern of marker expression at 3 days: rapid down-regulation of Ki-67 (indicating exit from the cell cycle) and nestin (suggesting differentiation into mature phenotypes). By 1 week, we saw the first evidence of GFAP expression, while GalC and NF200 where not seen until 2 weeks post treatment. By 4 weeks, Ki-67 was almost absent, and nestin was confined to a few long processes. GFAP and GalC were expressed in a low percentage of the cells, with approximately 20% expressing NF200. The remainder of the cells did not express any of the markers examined here.

Fate of Neural Progenitor Cells Placed Beneath the Kidney Capsule

Neural progenitor cells prepared from the brain of EGFP⁺ mice were placed beneath the kidney capsule of syngeneic C57BL/6 and allogeneic BALB/c recipients and
examined in situ at 1 hour, 7 days, 14 days, or 28 days after grafting by visual inspection through an operating microscope. All of the graft-bearing kidneys were then removed for immunohistochemical examination. At 1 hour and 1 week postgrafting, each heterotopic syngeneic and allogeneic graft of neural progenitor cells could be detected as a white mass beneath the kidney capsule, but the overlying capsule remained clear and nonvascularized. When these grafts were examined by confocal microscopy, EGFP+ cells could be easily detected (Fig. 1A and 1B). At 4 weeks, the grafts in both syngeneic and allogeneic recipients no longer appeared as a white mass. Instead, a nidus of EGFP+ cells was observed beneath the kidney capsule, again with no evidence of attendant neovascularization. At all observation points, both clinical and confocal microscopic appearances of the grafts in allogeneic recipients were indistinguishable from those in syngeneic recipients (Table 1). I-A\(^\beta\) and H-2K\(^\beta\) were not expressed in vitro, nor in the grafted cells placed beneath either allogeneic or syngeneic kidney capsules (Table 2). No evidence of rejection or necrosis of neural progenitor cell grafts was seen over the course of this study. This is in contrast to control grafts of freshly isolated neonatal cerebellum, where 75% of allografts placed beneath the kidney capsule were rejected in allogeneic hosts by day 28 (Table 1).

### Table 1. Fate of EGFP\(^+\) neural progenitor cell grafts placed under the kidney capsule of normal mice

<table>
<thead>
<tr>
<th>Donor/host</th>
<th>0</th>
<th>7 days</th>
<th>14 days</th>
<th>28 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progenitor cells/syngeneic</td>
<td>5/5</td>
<td>5/5</td>
<td>5/5</td>
<td>5/5</td>
</tr>
<tr>
<td>Progenitor cells/allogeneic</td>
<td>n.d.</td>
<td>5/5</td>
<td>n.d.</td>
<td>5/5</td>
</tr>
<tr>
<td>Neonatal cerebellum/syngeneic</td>
<td>n.d.</td>
<td>n.d.</td>
<td>5/5</td>
<td>5/5</td>
</tr>
<tr>
<td>Neonatal cerebellum/allogeneic</td>
<td>3/3</td>
<td>2/2</td>
<td>5/5</td>
<td>1/4</td>
</tr>
</tbody>
</table>

The survival of grafted progenitor or neonatal cerebellum grafts, placed in either syngeneic or allogeneic kidney capsules, demonstrates that progenitor grafts, but not neonatal cerebellum grafts, survive for 28 days in allogeneic hosts. n.d. = not done.
Grafted progenitor cells at this heterotopic site showed evidence of development along a neuronal lineage. Nestin expression, while quite high at the 7-day time point (Fig. 1A), decreased to undetectable levels by 28 days post-grafting (Fig. 1B). Neuronal development was first seen at 1 week, as shown by morphology (Fig. 1C, 1D, and 1E), and the expression of the neuron-specific markers microtubule-associated protein (MAP)-2 and MAP-5 (Fig. 1C; Table 2). Morphological differentiation was quite similar to that seen in homotopic progenitor cell grafts to sites in the CNS [12], with characteristic dendritic and axonal processes (Fig. 1C and 1D) and expression of appropriate neuronal markers (Fig. 1C).

The expression of GFAP was also detected in some cells within the grafts at 1 week, but at 28 days most of the cells developed neuronal morphology and marker expression patterns, and GFAP expression was decreased. No expression of oligodendrocyte markers was seen (data not shown).

Irrespective of whether CNS progenitor cells were placed beneath the kidney capsule of syngeneic or allogeneic recipients, no evidence of class I expression was detected over the course of this study (Fig. 1E and F; Table 2). Control grafts of neonatal cerebellum, however, showed clear staining for H-2Kb+ cells at 14 days postgrafting (Fig. 1G). Moreover, the pattern of morphological differentiation observed was virtually identical in allogeneic and syngeneic grafts. Importantly, we found no evidence, by morphology or by staining of CD45+ cells, of the presence of bone-marrow-derived cell lineages within progenitor cell grafts.

### Table 2. Summary of expression pattern seen in neural progenitor cells in vitro (under proliferation conditions) and under kidney capsule in vivo

<table>
<thead>
<tr>
<th>Marker</th>
<th>In vitro</th>
<th>Syngeneic 1 week</th>
<th>Syngeneic 4 weeks</th>
<th>Allogeneic 1 week</th>
<th>Allogeneic 4 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class I</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Class II</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>MAP-2</td>
<td>–</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>MAP-5</td>
<td>–</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Nestin</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>GFAP</td>
<td>–</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
</tbody>
</table>

While cells exposed to mitogens in vitro expressed only nestin, when grafted, they rapidly ceased to express this marker and instead turned on markers of more mature CNS-derived cells.

– = no expression; + = low expression; ++ = high expression.

The ability of allogeneic CNS progenitor cells implanted beneath the kidney capsule to take up residence and thrive without threat of immune rejection in normal mice indicates that the cells were immune privileged. It was important to determine whether this interesting property is unique to CNS progenitor cells, or if it is shared with organized CNS tissues. To examine this issue, neonatal cerebellar tissue was obtained from C57BL/6 mice and implanted beneath the kidney capsule of BALB/c and C57BL/6 recipients. The fate of these grafts was assessed clinically and by histologic and immunocytochemical examination at periodic intervals thereafter. As displayed in Table 1, syngeneic neonatal cerebellar tissue grafts accommodated to the subcapsular space of the kidney. These grafts appeared healthy and intact throughout the 28-day observation interval. Allogeneic neonatal cerebellar tissue grafts also accommodated to the subcapsular space of the kidney, displaying a healthy morphologic appearance at 7 days. Figure 1G shows grafts of neonatal cerebellum containing H-2Kb+ cells by 14 days postgrafting. During the next few weeks, these grafts became infiltrated with inflammatory cells and disintegrated. No GFP-expressing cells were found in three of four grafted kidneys, with the remaining graft appearing vastly diminished in size. On the one hand, these results indicate that neonatal CNS tissues can survive and maintain their morphologic integrity for prolonged intervals within the alien environment beneath the kidney capsule. On the other hand, when the neonatal CNS tissue is allogeneic to the recipient, the graft sensitizes its recipient and succumbs to immune rejection. Thus, the strong immune-privileged properties displayed by CNS progenitor cells are not shared by organized neonatal CNS tissue.

### Lack of Induction of Donor-Specific Delayed Hypersensitivity Following Neural Progenitor Cell Implantation

In order to determine whether allogeneic neural progenitor cell grafts had the capacity to sensitize recipients harboring these grafts beneath the kidney capsule, recipient mice were assayed for the acquisition of donor-specific delayed hypersensitivity (DH). EGFP+ neurospheres were implanted beneath the kidney capsules of normal BALB/c mice. As a positive immunizing control, additional BALB/c
mice instead received a s.c. injection of $10 \times 10^6$ C57BL/6 spleen cells. At 25 days after grafting, X-irradiated (2,000 R) C57BL/6 spleen cells ($1 \times 10^6$) were injected into the ear pinna. Ear swelling responses were assessed 24 and 48 hours later. The data of a representative experiment are presented in Figure 2A. Allogeneic neural progenitor cells beneath the kidney capsule failed to induce DH, in marked contrast to allogeneic spleen cells.

**Lack of Active Suppression of Donor-Specific DH Following Neural Progenitor Allograft Implantation**

To determine whether the failure of induction of allospecific DH was due to active suppression of DH or to a failure of allosensitization, the left ear pinnae of the same set of mice that had been subjected to DH assay at day 25 were rechallenged with irradiated C57BL/6 spleen cells at 32 days and ear swelling responses were assessed. Positive control BALB/c mice were immunized with right ear injections of $1 \times 10^6$ C57BL/6 spleen cells 1 week prior to the injection into left ear pinnae. Negative-control BALB/c mice received the left ear pinna injection alone. The results presented in Figure 2B indicate that the left ear pinna injection with irradiated C57BL/6 spleen cells at 1 week after the right ear pinna injection induced DH in mice receiving neural progenitor cell allografts. Thus, allogeneic neural progenitor cell grafts placed beneath the kidney capsule neither sensitized their recipients for DH nor rendered these mice incapable of becoming sensitized to donor alloantigens.

**Neural Progenitor Cells Present Alloantigens to Primed T Cells**

In order to determine whether neural progenitor cells are capable of expressing alloantigens, BALB/c mice were presensitized with allogeneic C57BL/6 spleen cells, after which their abilities to mount DH reactions when ear challenged with C57BL/6 neural progenitor cells were assessed. As revealed in Figure 2C, mice presensitized to C57BL/6 alloantigens developed significant ear-swelling responses compared with

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Figure 2. Neural progenitor cells lack immunogenicity. A) Lack of induction of donor-specific delayed hypersensitivity. We evaluated for the induction of DH following implantation of CNS progenitor cell allografts beneath the kidney capsule of BALB/c mice at 25 days. Positive immunization controls (immunized) received s.c. injection of $10 \times 10^6$ donor-spleen cells 1 week prior to assay. Right ear pinna received injection of X-irradiated C57BL/6 spleen cells ($1 \times 10^6$), and ear swelling responses were assessed 24 and 48 hours later. Positive (immunized) and negative (naïve) controls are similar to those described in legend to Figure 3A. Mean ear swelling responses are compared with negative controls. * = mean value significantly greater than negative control, $p < 0.001$. (B) Lack of active suppression of donor-specific DH. Donor-specific DH at 24 hours after initial ear challenge following implantation of neural progenitor cell allografts beneath the kidney capsule of allogeneic BALB/c mice. Left ear of the recipient mice received injection of X-irradiated C57BL/6 spleen cells ($1 \times 10^6$), and ear swelling responses were assessed 24 and 48 hours later. Positive (immunized) and negative (naïve) controls are similar to those described in legend to Figure 3A. Mean ear swelling responses are compared with negative controls. * = mean value significantly greater than negative control, $p < 0.001$. (C) Neural progenitor cells present alloantigens to primed T cells. Elicitation of donor-specific DH specific in BALB/c mice by injection of C57BL/6 neural progenitor cells, following recipient presensitization with C57BL/6 spleen cells. Both test mice and positive controls first received s.c. immunization with $10 \times 10^6$ C57BL/6 spleen cells. One week later, positive (immunized) and negative (naïve) control mice received ear pinna challenge with $10 \mu l$ of X-irradiated C57BL/6 spleen cells ($1 \times 10^6$), whereas test mice received ear pinna challenge with X-irradiated neural progenitor cells ($10 \mu l$ of $1 \times 10^6$). Ear swelling responses were measured at 24 and 48 hours. Mean ear swelling responses (±SEM) at 24 hours are presented. * = mean values greater than negative control, $p < 0.01$. 

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negative controls. This indicates that neural progenitor cells display histocompatibility antigens in a manner that permits presensitized T cells to recognize and respond to the cells.

**Fate of Neural Progenitor Cells Beneath the Kidney Capsules of Pre- or Postsensitized Mice**

Our next experiments were designed to determine whether neural progenitor cells can be a target of alloimmune rejection. The graft-bearing kidneys of BALB/c mice that had been challenged with C57BL/6 spleen cells in the right ear at 25 days and in the left ear at 32 days were harvested at 42 days, and the fates of the grafts were observed by confocal microscopy. Results revealed that none of the recipient kidneys contained EGFP+ cells. Instead, CD45+ cells accumulated at the graft site in all samples, indicating that EGFP+ neural progenitor cells had been eliminated after ear challenge with C57BL/6-spleen cells (Fig. 3).

To confirm the capacity of neural progenitor cells to be a target of alloimmune rejection, the allogeneic neural progenitor cells were placed beneath the kidney capsule of presensitized mice. Normal BALB/c mice were immunized s.c. with $10 \times 10^6$ C57BL/6 spleen cells. One week later, EGFP+ neurospheres were placed beneath the kidney capsules of these presensitized mice. Thirteen days later, the graft-containing kidneys were inspected clinically, then removed and examined by confocal microscopy. By clinical inspection, none of these kidneys contained the white mass or transparent nidus characteristic of a surviving graft. Moreover, confocal microscopy showed no EGFP+ cells in any samples, but instead revealed an accumulation of CD45+ cells at the graft site. Thus, allogeneic neural progenitor cells, incapable of sensitizing recipients, were nonetheless vulnerable to rejection in specifically sensitized recipients—whether sensitization preceded engraftment or took place after the graft had become established at its heterotopic site (Table 3).

**Expression of MHC Antigens by Cultured CNS Progenitor Cells: Baseline versus Stimulation with IFN-γ**

When studied using flow cytometric analysis, CNS progenitor cells did not express detectable class I (H-2Kβ, β-2 microglobulin) or class II (I-Aβ) MHC antigens when cultured under standard proliferation conditions (Fig. 4). This lack of expression persisted under differentiation conditions (10% FCS for 8 days) known to induce marked morphological changes in cultured CNS progenitor cells as well as expression of the mature markers MAP-2, MAP-5, GFAP, and GalC. The lack of MHC expression detectable by flow cytometry therefore confirms the negative immunocytochemical data, both in vitro and in vivo. Expression of class I and class II MHC antigens was readily induced in vitro by the addition of IFN-γ and reversed by subsequent withdrawal of that cytokine, demonstrating that lack of baseline expression was not a result of an inability to express these antigens.

**DISCUSSION**

Progenitor cells harvested from the CNS of EGFP transgenic mice display the properties of immune-privileged tissues. The validity of this assertion rests on the results presented in this communication. When implanted at a non-immune-privileged site, such as beneath the kidney capsule, both syngeneic and allogeneic CNS progenitor cells established residence and carried out a recognizable version of

<table>
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<tr>
<th>Table 3. Fate of EGFP+ neural progenitor cell grafts placed under the kidney capsules of pre- or post-sensitized mice</th>
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</thead>
<tbody>
<tr>
<td><strong>Recipient (BALB/c)</strong></td>
</tr>
<tr>
<td>Presensitized (-7 days)</td>
</tr>
<tr>
<td>Postsensitized (25 + 32 days)</td>
</tr>
<tr>
<td>Naïve</td>
</tr>
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*Sensitization with respect to graft placement beneath kidney capsule, which is at time = 0.

Animals injected with spleen cells from the same strain as the donor progenitor cells rejected the progenitor cell grafts and did so whether they received the spleen cell injections before (pre-sensitized) or after (post-sensitized) the progenitor cell grafts.
Figure 4. Dynamic regulation of MHC expression. Cultured CNS progenitor cells did not express MHC antigens when grown under proliferation conditions (EGF), and the absence of detectable MHC expression was not altered by differentiation conditions (FCS 10% × 8 days). In contrast, both class I (H2Kb, β2 microglobulin) and class II (I-A') MHC antigens were induced by the addition of murine recombinant IFN-γ, appearing by day 3 and reaching high levels on day 4, before returning to negative baseline expression following subsequent IFN-γ withdrawal. Dark profiles = molecule of interest; unfilled lines = isotype control.
their development program by differentiating into cells with neural and glial phenotypes. Since allogeneic CNS progenitor cells formed stable grafts that continued to thrive for at least 4 weeks, and since allogeneic neonatal cerebellar grafts had been destroyed at that time, CNS progenitor cells displayed the properties of an immune-privileged tissue. In support of this interpretation are the following experimental findings: CNS progenitor cells did not display class I or II MHC antigens either in culture or as grafts beneath the kidney capsule. Moreover, allogeneic CNS progenitor cells did not induce donor-specific DH in mice bearing CNS progenitor cell grafts beneath the kidney capsule. The facts that cognate sensitization of recipients to donor alloantigens prevented allogeneic CNS progenitor cells from establishing residence beneath the kidney capsule and caused the elimination of established CNS progenitor cell grafts indicate that CNS progenitor cells do in fact express transplantation antigens. Since allogeneic CNS progenitor cell grafts proved incapable of sensitizing their recipients, they lack the property of allograft immunogenicity but retain the property of alloantigenicity.

As a site for the study of the transplantation immunology of solid tissue grafts, the subcapsular sinus of the kidney has great value. Developed in the 1970s as a method for examining the immunogenicity of solid tissue grafts, the subcapsular kidney sinus has helped investigators to understand the immunogenicity of allogeneic skin, heart, liver, and islets of Langerhans [10]. Beligrau et al. reawakened interest in the subcapsular sinus of the kidney in 1995 by reporting that this “conventional” (non-immune-privileged) site could be used to demonstrate that allografts of testis possess the property of an immune-privileged tissue [3]. These sites manifest immune privilege through a number of distinct mechanisms, including Fas/FasL tolerance induction and acquired immune deviation, among others. Our laboratory has taken advantage of this approach by demonstrating that retinal pigment epithelium [14], as well as epithelium-deprived corneal tissue [13], function as immune-privileged tissues when placed beneath the kidney capsule. The present experiments extend these studies to include progenitor cells derived from mammalian CNS, documenting the immune—privileged status of these unique and promising cells.

We suggest that, while we have taken great pains to avoid the use of the term neural stem cells, this is fundamentally a semantic issue. To our knowledge there have been no published studies of “pure” neural stem cell transplants (i.e., all grafts contain a mixed population of stem and more committed progenitor cells) as we describe in this work. We find it very encouraging that such a mixed population of cells maintains such a high level of immune privilege. This suggests that, at least in terms of the immune response, one does not require a pure stem cell population as a source of donor tissue, and that somewhat committed, and therefore differentiated, neural progenitor cells still possess inherent immune privilege. Whether the ability of the grafted cells to survive, differentiate, and functionally integrate with the host will require a pure population of stem cells remains to be determined.

The finding that allogeneic CNS progenitor cells were unable to survive in recipients sensitized systemically to donor alloantigens deserves special comment. The terms “immunogenic” and “antigenic,” when applied to tissue transplants, indicate respectively: A) the ability of an allograft to sensitize its recipient, and B) the vulnerability of the graft to specific immune effectors of rejection. Allogeneic skin grafts placed beneath the kidney capsule display both immunogenicity and antigenicity. By contrast, our results indicate that similarly implanted allogeneic CNS progenitor cells lack immunogenicity but retain antigenicity.

There are several possible explanations for this dichotomy between immunogenicity and antigenicity with respect to neural progenitor cells, including lack of costimulatory molecules, secretion of immunosuppressive factors, and unwinding induction of donor-specific tolerance. Our results do not permit us to speculate that any one of these factors is relevant in this situation. It is, however, important to point out that we have shown that CNS progenitor cells do not induce DH suppressor cells in recipient mice. This implies that the lack of immunogenicity among neural progenitor cells does not result from the induction of tolerance.

In the case of skin grafts, much, if not all, of the immunogenicity resides in its complement of mobile bone marrow-derived cells (chiefly dendritic cells and macrophages, passenger leukocytes) that constitutively express high levels of MHC class I and II molecules, along with potent costimulatory signals. CNS progenitor cell grafts lack passenger cells at the time of implantation, and our evidence indicates that CD45+ cells derived from progenitor cell progenitors did not emerge within these grafts. Although there is evidence for the differentiation of neural progenitor cells into cells of bone marrow origin, this has only been reported within a specific experimental paradigm (sublethal whole-body irradiation, grafting to the blood stream) [15]. Our control experiment, in which neonatal cerebellar grafts were rapidly rejected in the subcapsular space, offers some insight into the mechanisms of progenitor cell graft acceptance. One major difference between these two types of grafts is the presence of passenger leukocytes capable of sensitizing the host and inducing graft rejection. More studies are needed to determine if this mechanism underlies progenitor cell graft survival (or indeed neonatal graft rejection), but at present, it appears to be a viable hypothesis.

It is clear that CNS progenitor cells can differentiate into various lineages, including glial cells, and this has been demonstrated both in vivo and in vitro. We, together with
many others, have shown that upon exposure to serum in culture, or upon transplantation to the CNS, neural progenitor cells differentiate into neurons, astrocytes, and oligodendrocytes, the relative proportion of a particular phenotype being influenced by the CNS compartment chosen as the recipient site [9, 12]. Here, we show that 1 week after grafting under the kidney capsule, neural progenitor cells express both neuronal and astrocytic markers. In neither instance did we find evidence of class I MHC expression. Expression of class I molecules in astrocytes is viewed differently by different authors. On one hand, it is well known that mature astrocytes constitutively express class I MHC antigen, albeit at relatively low levels [16], and can, therefore, serve as targets for class I-specific cytotoxicity, even when class I expression is undetectable by immunocytochemistry [17]. Astrocytes can be induced to upregulate class I levels and to express class II antigens, but only following in vitro treatment with agents such as IFN-γ or tumor necrosis factor-α [18, 19], or, in vivo, as a result of a wide range of neuropathological processes in which such agents likely play a role [20]. On the other hand, there is also evidence that resting astrocytes actively contribute to immune privilege in the CNS, in part by secreting factors that downregulate the expression of MHC [21] on monocytes and by interfering with the activation of primary CD4+ T cells [22]. Moreover, resting astrocytes are unable to serve as antigen-presenting cells in vivo [23].

In this study, we found no direct evidence for expression of MHC antigens by the mouse neural progenitor cells or their differentiated progeny, except when such expression was induced in vitro using IFN-γ. These data are consistent with our transplantation results showing grafted CNS progenitor cells to be nonimmunogenic when transplanted to a conventional site. Alternatively, the rapid and complete rejection of CNS progenitor cells from beneath the kidney capsule following peripheral immunization with allogeneic spleen cells from identical donors indicates that the donor neural progenitor cells exhibited nonimmunogenic antigenicity. The identity of the antigens initiating this rejection remains to be elucidated. One possibility is that MHC class I antigens were expressed at levels below the threshold of detection by either immunocytochemistry or flow cytometry [24]. Alternatively, minor transplantation antigens may be the target of the rejection response. Thus, the lack of immunogenicity we found in CNS progenitor cell allografts beneath the kidney capsule is no guarantee of universal acceptance of these grafts under any circumstance. Our evidence indicates that exposure to proinflammatory cytokines or the pre-existence of donor-specific immunity within the recipient can render the graft vulnerable to rejection.

Together with the virtual absence of MHC alloantigens on CNS progenitor cells, the lack of immunogenicity of these grafts is understandable. It is relevant that allogeneic neonatal cerebellar grafts, which do contain passenger cells in the form of microglia, suffered a different fate from allogeneic CNS progenitor cell grafts. We presented evidence previously that microglia within neonatal neuronal retinal grafts display properties similar to passenger leukocytes [25]. We suspect that microglia within the neonatal cerebellar grafts placed beneath the kidney capsule alerted the recipient immune system to the existence of the graft, thereby initiating its eventual rejection.

Recent evidence that human embryonic stem [26] and embryonic germ [27] cells possess the intrinsic developmental capacity of pluripotent stem cells has generated considerable interest in the burgeoning field of regenerative medicine. Results from stem and progenitor cell transplantation experiments, in a variety of paradigms, suggest to us that it is worth revisiting the historical concepts of plasticity, fate commitment, and lineage determination [15, 28, 29]. In particular, the ability of grafted progenitor cells to respond appropriately to injury within the mature CNS [8, 9] has greatly heightened the expectation that these cells could play a role in tissue repair and cell replacement in a clinical setting. However, a number of fundamental issues in stem cell biology need to be resolved before such applications should be considered. While cell migration, integration, and proliferation are important aspects of stem cell development in need of study, we would submit that the intrinsic immunological properties of donor progenitor cells are also among the chief concerns regarding transplantation of these cells into human patients.

After more than a decade of human neural transplantation studies [30-32], our knowledge of the basic immunological properties of conventional embryonic and fetal donor tissue remains inadequate. In most cases, immunological concerns are not specifically addressed, with immunosuppressive drugs being applied to elderly patients suffering from neurodegenerative diseases undergoing highly invasive neurosurgical procedures. The results we present here demonstrate that neuronal progenitor cells are a nonimmunogenic immune-privileged tissue, and that they can be grafted into allogeneic recipients without the need to impose potentially toxic immunosuppressive regimens. These results are encouraging with respect to the ultimate immunological success of neural progenitor cell transplantation.

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