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The immunological properties of adult hippocampal progenitor cells

Henry Klassen [a,*], Karen L. Imfeld [b], Jasodhara Ray [c], Michael J. Young [d], Fred H. Gage [c], Monique A. Berman [b]

[a] *Stem Cell Research, Children's Hospital of Orange County, 455 South Main Street, Orange, CA 92868, USA*
[b] *Center for Research and Treatment of Immune and Inflammatory Diseases, Children's Hospital of Orange County, 455 South Main Street, Orange, CA 92868, USA*
[c] *Laboratory of Genetics, Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, CA 92037, USA*
[d] *Schepens Eye Research Institute, Department of Ophthalmology, Harvard Medical School, 20 Staniford Street, Boston, MA 02114, USA*

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Abstract

Adult hippocampal progenitor cells (AHPCs) derived from mature rats were studied in mixed co-cultures and shown not to elicit a proliferative response from human peripheral blood mononuclear cells (PBMCs) or allogeneic spleen cells. FACS analysis revealed low class I and no detectable class II (Ia) MHC expression by these cells. RT-PCR showed that AHPCs express the anti-inflammatory cytokine TGF-β1. AHPCs did not, however, significantly impede the proliferation of OKT3- or PHA-stimulated PBMCs. Taken together, these results indicate that AHPCs are non-immunogenic in vitro. This is consistent with their pattern of MHC expression and does not require an active immunosuppressive mechanism.

Keywords: Neural stem cells; Neuroimmunology; MHC expression; Rat

1. Introduction

Multipotent neural progenitor cell lines have attracted considerable interest recently, particularly as sources of highly plastic donor cells for transplantation studies (Gage, Ray, & Fisher, 1995; Renfranz, Cunningham, & McKay, 1991; Sabate et al., 1995; Snyder et al., 1992; Young, Ray, Whiteley, Klassen, & Gage, 2000). Although neural progenitor cells frequently survive grafting to non-syngeneic hosts (Brustle et al., 1998; Flax et al., 1998; Fricker et al., 1999; McDonald et al., 1999; Mizumoto et al., 2001; Shatos et al., 2001; Young et al., 2000) transplantation has generally targeted compartments of the central nervous system (CNS) where the phenomenon of immune privilege is known to apply (Brevig, Holgersson, & Widner, 2000; Jiang & Streilein, 1994; Poltorak & Freed, 1991; Schnell, Fearn, Klassen, Schwab, & Perry, 1999; Wenkel, Streilein, & Young, 2000). The transplant immunology of neural progenitor cells has not been specifically investigated and therefore it has been unclear whether these cells exhibit decreased immunogenicity when grafted outside of the CNS microenvironment. One reason to believe they might comes from studies of the neuronal and glial populations comprising the CNS parenchyma. The cells of the mature CNS express low levels of MHC antigens under normal conditions (Rall, 1998) and therefore display decreased immunogenicity (Aloisi et al., 1999; Wucherpfennig, 1994). Nevertheless, it is known that CNS progenitors differ markedly from their mature progeny with respect to a range of molecular markers. Whether neural progenitors share the immunological characteristics of differentiated CNS cells is not known.

Cells capable of generating new neurons have long been known to reside in specific sites within the adult CNS of rodent (Altman & Das, 1965) and avian (Goldman & Nottebohm, 1983) species. Until recently, however, these cells had not been successfully propagated ex vivo. The advent of an abundant supply of recombinant mitogens, notably basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF), has allowed the isolation of multipotent neural progenitors from CNS tissue via selective proliferation (Reynolds & Weiss, 1992) or following automated cell sorting

* Corresponding author. Tel.: +1-714-516-4280; fax: +1-714-289-4531.
E-mail address: hklassen@choc.org (H. Klassen).

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(Uchida et al., 2000). Although frequently obtained from embryonic sources, neural progenitor cells can also be derived from adult rodents. For example, adult hippocampal progenitor cells (AHPCs) have been clonally-derived from the dentate gyrus of rats and mice and expanded over many generations in vitro (Gage & Ray et al.). In the adult rodent, cells born in the subventricular zone (SVZ) are known to enter the rostral migratory stream leading to the olfactory bulb where they differentiate into resident neurons (Lois & Alvarez-Buylla, 1993). Recently, it has been demonstrated that proliferation-competent cells are also present in the SVZ and dentate gyrus of aged humans (Eriksson et al., 1998). Although the physiological significance of such cells in humans is not yet known, their mere existence has generated considerable interest. In particular, neural progenitor cells are of value for transplantation studies directed towards neuronal repopulation of the injured or degenerating CNS. Transplanted stem or progenitor cells appear to be capable of migrating into and repopulating a range of tissues, including brain (Brustle et al., 1998) retina (Takahashi, Palmer, Takahashi, & Gage, 1998; Young et al., 2000) spinal cord (Cao et al., 2001; McDonald et al., 1999) and perhaps bone marrow (Bjornson, Rietze, Reynolds, Magli, & Vescovi, 1999).

The preceding experiments included an element of immunoprotection that may have contributed to graft survival, namely, CNS-associated immune privilege or systemic marrow ablation. The questions of whether neural progenitor cells can themselves avoid immune detection, or induce immune deviation, have not been addressed. To assess the cell-mediated response elicited by AHPCs in vitro, we combined these cells with peripheral blood mononuclear cells (PBMCs) or spleen cells in one-way mixed cultures.

2. Materials and methods

2.1. Neural progenitor cell line

AHPCs were originally isolated from the brain of adult female Fischer 344 rats, as previously described (Gage et al., 1995). Briefly, rats were anesthetized, decapitated and the hippocampal formation dissected free. Dissected hippocampal tissue was incubated with enzymes (papain, neutral protease, DNAse) and the resulting cell suspension was washed and incubated in serum-containing medium for 24 h, followed by incubation in a defined medium containing high levels of bFGF (FGF-2). From these proliferating cells, clonal isolates were obtained and transfected via a retroviral construct (Sah, Ray, & Gage, 1997) to express the enhanced jellyfish green fluorescent protein (eGFP) reporter gene. Because our previous intraocular transplantation study used an eGFP expressing clone (Young et al., 2000) the same was used in the present study.

As previously described, AHPCs were grown as an adherent monolayer in tissue culture flasks coated with polyornithine/laminin and containing DMEM/F-12 (Omega Scientific, Tarzana, CA) supplemented with 1 mM L-glutamine (Irvine Scientific, Santa Ana, CA), N2 Supplement (Invitrogen, Carlsbad, CA) and human recombinant bFGF (FGF-2, Promega, Madison, WI) at 20 ng/ml. Medium was completed replaced every 2–3 days and cultures split prior to reaching confluence. AHPCs were harvested using a trypsin/EDTA solution (Custom ATV, Irvine Scientific), washed in DMEM/F-12, centrifuged (1000 g, 3 min), resuspended and yield assessed using trypan blue hemocytometry. Specifically for plating into co-cultures, AHPCs were resuspended in AIM V media without additional growth factors.

In specific cases, AHPCs were grown under differentiating conditions, as above but without added bFGF and in the presence of 5% fetal bovine serum (FBS, Irvine Scientific). In other cases, AHPCs were grown under MHC antigen-inducing conditions consisting of standard proliferation medium supplemented with recombinant rat IFN-γ (100 ng/ml, R&D Systems, Minneapolis, MN). After 4 days of treatment, IFN-γ was withdrawn via complete change to fresh standard proliferation medium.

2.2. Human peripheral blood mononuclear cells

Between 50–100 ml peripheral venous blood was drawn, per experiment, from a human volunteer (42 yr old male; 51 yr old female) and collected in heparinized tubes. Mononuclear cells were isolated by centrifugation in Ficoll-Paque (Pharmacia, Peapack, NJ), washed in HBSS (Invitrogen, Carlsbad, CA) and resuspended in AIM V medium (Invitrogen).

2.3. Rat spleen cells

Spleens were dissected from adult female rats (syngeneic = Fischer 344, allogeneic = Sprague Dawley) after sacrifice via CO_2 atmosphere. Spleens were minced and extruded through a small pore filter to separate cells. Resulting cell suspension was treated similarly to PBMCs, but without Ficoll-Paque.

2.4. Mitomycin C treatment

The resuspended cells, both AHPCs and PBMCs, were divided into treatment groups prior to being plated to culture wells. Cells to be treated (AHPCs, PMBCs, or spleen cells) were incubated in mitomycin C (Sigma) 25 μg/ml at 37 °C in the dark for 20 min, then washed in fresh media ×3 with centrifugation and recounted.

2.5. Mixed co-cultures

Both mitomycin-treated and untreated AHPCs were diluted to 5×10^5 cells/ml in AIM V medium and aliquoted into wells on 96 well plates (Greiner, Frickenhausen, Germany) at 100 μl/well. PBMCs and spleen cells were diluted to 3×10^6 cells/ml before being plated at 100 μl/well. After plating was complete, those wells containing only a single cell type received an additional 100 μl of AIM V such that all wells contained 200 μl final volume. In specific experiments, FBS was included in all wells at a concentration of 5%. Co-cultures were organized into eight distinct well types representing every solitary or paired permutation of AHPC and PBMC, treatment group included (e.g., Fig. 1). To provide a statistical base, each data point (well type) represented 6, or more, individual wells. Subsets of co-culture plates were frozen on day 2 or 4 for cytokine analysis.

2.6. Control co-cultures

As a positive control to demonstrate the ability of human PBMCs to proliferate in the presence of rat tissue, mixed co-cultures with rat spleen cells (Sprague-Dawley) were performed. As a negative control to demonstrate a lack of response to AHPCs by syngeneic effector cells, mixed co-cultures with rat spleen cells (Fischer 344) were also carried out.

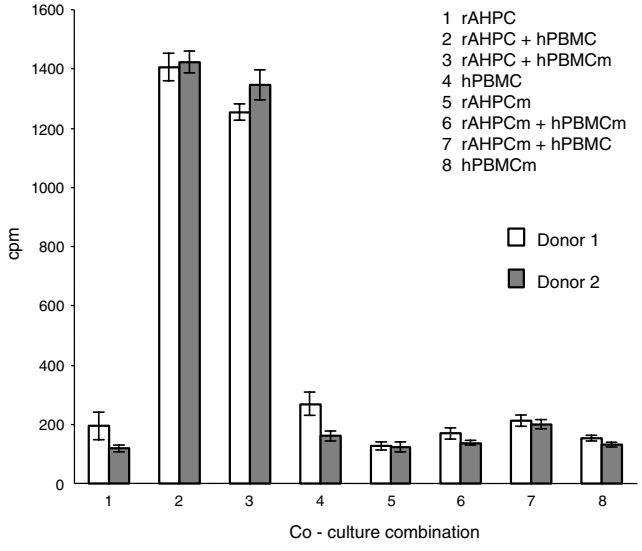

Fig. 1. Proliferation study of Fischer 344 rat AHPCs and human PBMCs in mixed co-cultures, with comparison between different PBMC donors. Note the lack of response for co-culture combination 7, indicating that hPBMCs do not proliferate in response to mitomycin-treated rAHPCs. In contrast, combinations 2 and 3 show proliferation of rAHPCs in response to treated or untreated hPBMCs. The co-culture profile shown here was highly reproducible and was not dependent on PBMC donor. Shown are mean and SEM of pooled data from two experiments, each with triplicate determinations at each position for each donor. [^{3}H]TdR was added 24 h prior to harvest on day 6. The suffix 'm' indicates cells treated with mitomycin C.

2.7. Cytokine detection

Subset of co-culture plates were frozen on day 2 or 4 for cytokine analysis. These plates were later thawed and supernatants and cell lysates processed for ELISA for human IFN-γ or IL-4 (Biosource, Camarillo, CA). The working range was approximately 10–10,000 pg/ml.

2.8. Direct mitogenic stimulation of PBMCs

In one experiment, PBMCs were cultured in the presence of reagents known to provide antigen-independent stimulation of lymphocyte proliferation. Either OKT3 cell-conditioned supernatant or PHA (Bacto-Phytohemagglutinin P, 50 μl of 1% rehydrated, Difco Laboratories, Detroit, MI) were added to a subset of wells on 96 well plates containing either PBMCs, AHPCs, or both, prepared as described previously. Wells were radio-labeled on day 1 or 3 and harvested 1 day later (day 2 or 4).

2.9. Mitogenic stimulation of AHPCs by PBMC-conditioned medium

To investigate the stimulatory effect of human leukocytes on rat neural progenitor cells, AHPCs were cultured without supplemental bFGF in medium supplemented with supernatant conditioned by PBMCs. Cells were grown on 96 well plates in 200 μl Aim V medium. After 5 days, cell-free supernatants were harvested from PBMC wells and transferred to freshly plated AHPCs as aliquots of 7.5, 15, or 75 μl. Wells were labeled with [^{3}H]TdR on day 1, harvested on day 5, and processed for scintillometry.

2.10. Scintillometry

Co-culture plates not used for cytokine analysis were labeled with 0.5 μCi [^{3}H]TdR (6.7 Ci/mM, Amersham) on either day 5 or day 8 and frozen one day later. After thawing, all labeled wells were scraped to assure suspension of organic material and harvested onto glass microfiber filters (Whatman, Fairfield, NJ) using a Cell Harvester Model M-24V (Brandel, Rockville, MD). Filters were placed into vials that were then filled with 2 ml of Biosafe II (RPI) and [^{3}H]TdR activity counted using a Beckman LS 1800 Scintillation Counter (Brea,

CA). Mean cpm of replicate samples (6 or greater) was determined.

2.11. Statistical analysis

Scintillation data from mixed co-culture experiments were initially analyzed using the Kruskal–Wallis test to detect the presence of a significant result, followed by pair-wise analysis of relevant data subsets using the Wilcoxon (Mann–Whitney) test.

2.12. Flow cytometry

AHPCs or rat spleen cells were harvested as described above and resuspended in PBS as a single cell suspension at 10^7 cells/ml. Cells were then transferred in 100 μl aliquots to individual round-bottom 12×75 mm polystyrene centrifuge tubes for specific labeling. Multiparameter cytometry was performed on FACS Vantage (Becton Dickinson, San Jose, CA) incorporating a Macintosh G3 computer (Apple, Cupertino, CA) running proprietary software.

To examine MHC antigens, the primary antibodies used were purified IgG1 mouse anti-rat monoclonals (Serotec, Kidlington, UK) used at 5 μg/100 μl. OX-18 was used to label MHC class I through binding to the RT-1A monomorphic determinant, OX-6 was used to label MHC class II through binding to a monomorphic determinant of the I-A antigen. Mouse IgG1, κ (MOPC-21, Sigma, St. Louis, MO) was used as isotype control (5 μg/100 μl), phycoerythrin (PE)-conjugated $F(ab)_2$ fragment of sheep anti-mouse IgG (Sigma) was used as secondary (5 μg into residual volume after decanting). To test for the presence of Fas Ligand (CD95L) on AHPCs, a biotin-conjugated hamster anti-mouse monoclonal primary with known cross-reactivity to rat FasL (PharMingen, San Diego, CA) was used at 5 μg/100 μl, followed by Cy-Chrome streptavidin secondary (PharMingen) 0.06 μg/10^6 cells.

2.13. RT–PCR

Total RNA was isolated with Trizol Reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Two micrograms of RNA were reverse transcribed into cDNA using the First-Strand cDNA Synthesis Kit (Pharmacia). The cDNA was then used for multiplex PCR using CytoXpress Quantitative PCR Detection Kit (BioSource). The rat Th1/Th2 Switch Cytokine and Rat Inflammatory Cytokine Set 1 were used. A sample consisting of 20 μg of PCR reaction product was then analyzed by gel electrophoresis and visualized by ethidium bromide (EtBr) staining.

3. Results

3.1. AHPCs proliferate in mixed co-cultures, PBMCs do not

Recognition of MHC disparate cells by lymphocytes leads to their proliferation and generation of cytotoxic T cells (T_C) with subsequent graft rejection. To examine the immunogenicity of neural stem cells, we used a line AHPCs to challenge co-cultured human PBMCs in vitro. Cells were either used untreated or pre-treated with mitomycin C to block proliferation. Cultures were maintained for a pre-determined number of days and [^{3}H]TdR was added 24 h prior to harvest.

Mixed co-cultures harvested at day 6 consistently showed significant differences in [^{3}H]TdR uptake between combinatorial wells, indicating non-uniformity of proliferative responses (Kruskal–Wallis, $P > 0.0001$) (Fig. 1). Further analysis (Wilcoxon Rank Sum) confirmed the basis of this non-uniformity to be increased proliferation in wells containing untreated AHPCs in combination with PBMCs, regardless of whether the latter were treated or not. This indicated that proliferation of AHPCs, and not PBMCs, was responsible for the increased [^{3}H]TdR uptake seen. The requirement for PBMCs suggested that these cells exert a mitogenic effect on neural progenitors.

Conversely, mitomycin-treated AHPCs did not elicit a proliferative response from either untreated PBMCs. These results suggest that AHPCs either do not provide non-syngeneic mononuclear cells with an immunogenic stimulus or actively impede PBMC proliferation following immunogenic stimulation. Co-culture findings were reproducible using unrelated PBMC donors (Fig. 1).

3.2. Lack of PBMC proliferation extends to a range of co-culture conditions

To further delineate the lack of mononuclear cell response to AHPCs, additional experiments were performed. Harvesting of co-cultures at multiple time points from 2 to 7 days showed that the absence of PBMC proliferation was a general phenomenon and not specific to the 6 day time point (Fig. 2). Similar results were obtained when AHPCs were co-cultured with allogeneic spleen cells instead of xenogeneic PBMCs (Fig. 2).

As a positive control for responsiveness to rat immunogens, human PBMCs were challenged with rat spleen cells. Mitomycin-treated xenogeneic spleen cells generated a proliferative response in co-cultured PMBCs (Fig. 2). As a negative control, Fischer 344 rat spleen cells were challenged with AHPCs. No proliferative response to the syngeneic progenitors was seen (Fig. 2). Similar to the mitogenic influence of human

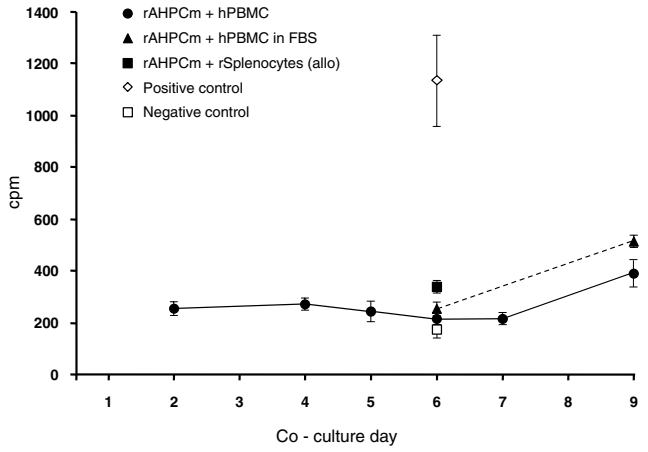

Fig. 2. Summary of co-culture data. Filled circles connected by solid line illustrate the inability of rAHPCs to induce hPBMC proliferation across a range of time points. Similar results were obtained from co-cultures containing FBS (filled triangles, broken line) as well as from allogeneic co-cultures using Sprague Dawley rat spleen cells instead of hPBMCs (filled square). Positive control verifies proliferation of hPBMCs in response to Fischer 344 rat spleen cells syngeneic to rAHPCs (open diamond). Negative control shows no response of syngeneic spleen cells to rAHPCs (open square). In each case, [^{3}H]TdR was added 24 h prior to harvest. Mean, SEM.

PBMCs, sygeneic rat spleen cells exerted a trophic effect on AHPCs (Wilcoxon Rank Sum, $P = 0.0039$).

To investigate the possibility of a late response (Murray, Nelson, Rayat, Elliott, & Korbutt, 1999), AHPCs and PBMCs were co-cultured for 9 days. Results at this time point were also consistent with the lack of an immunogenic response (Fig. 2). The moderate elevation in [^{3}H]TdR uptake by co-cultures of mitomycin-treated AHPCs and untreated PBMCs (Fig. 2) was significantly less than the marked elevation seen with positive controls (Wilcoxon Rank Sum, $P = 0.0001$).

An established means of promoting the differentiation of hippocampal progenitors is exposing them to serum (Ray, Peterson, Schinstine, & Gage, 1993). To avoid this, experiments up to this point were performed using serum-free conditions. However, because AHPCs express mature neuronal markers following transplantation (Young et al., 2000), it was of interest to know whether the immunogenicity of AHPCs is influenced by differentiation. Initial studies confirmed widespread expression of MAPs and of GFAP by AHPCs after 5 days incubation in vitro in FBS-containing media. Mixed co-cultures of AHPCs and PBMCs in FBS were harvested on day 6 or day 9 (Fig. 2). The presence of serum had a mitogenic effect on untreated AHPCs. Treated AHPCs, however, were not associated with proliferation of co-cultured PBMCs at the 6 day time point. At the later 9 day time point [^{3}H]TdR uptake was moderately elevated, similar to results obtained at 9 days without FBS and again significantly less than positive controls.

3.3. Cytokine production in co-cultures

The production of specific cytokine profiles has been associated with different types of immune response. As part of our evaluation of whether AHPCs stimulate xenogeneic PBMCs, mixed co-cultures were assayed for the production of selected cytokines. Particular attention was given to IFN-γ, an important indicator of a Th1-type lymphoproliferative response (Fig. 3). Levels of hIFN-γ were not elevated beyond background levels in wells containing PBMCs, either alone or co-cultured with AHPCs, nor was there any cross-reactivity in wells containing AHPCs alone. In contrast, high levels of IFN-γ were seen in wells containing OKT3- or PHA-stimulated PBMCs. Similarly, ELISA data for IL-4, a cytokine indicative of a Th2-type response, did not shown elevation beyond background levels in wells containing PBMCs or AHPCs, either alone or in combination (all less than 5 pg/ml).

3.4. Expression of immune-related surface molecules by AHPCs

One means of decreasing immunogenicity without the need for active inhibition of lymphocytes is through decreased expression of transplantation antigens, particularly molecules of the major histocompatibility complex. Flow cytometry was used to evaluate the expression of both class I and class II (I-a) rat MHC molecules (Fig. 4). Class I MHC was detected on AHPCs, although baseline expression of this antigen was

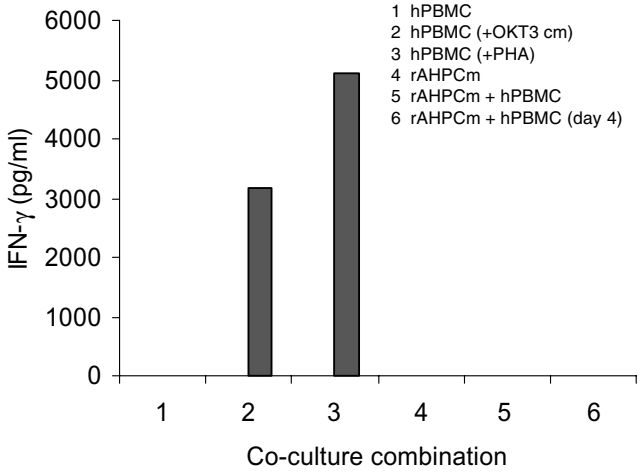

Fig. 3. Stimulation of IFN-γ production by PBMCs. Cultured hPBMCs do not produce appreciable (>10 pg/ml) IFN-γ unless stimulated to do so, as is seen here with the addition of OKT3 cell-conditioned medium or PHA. The presence of mitomycin-treated rAHPCs does not stimulate IFN-γ production by hPBMCs in co-culture, supporting the lack of a Th1-type cytokine response. Data for PBMC and mitomycin-treated AHPC controls were equivalent at days 2 and 4 (data shown is from day 2, unless otherwise indicated). Measured by ELISA against hIFN-γ.

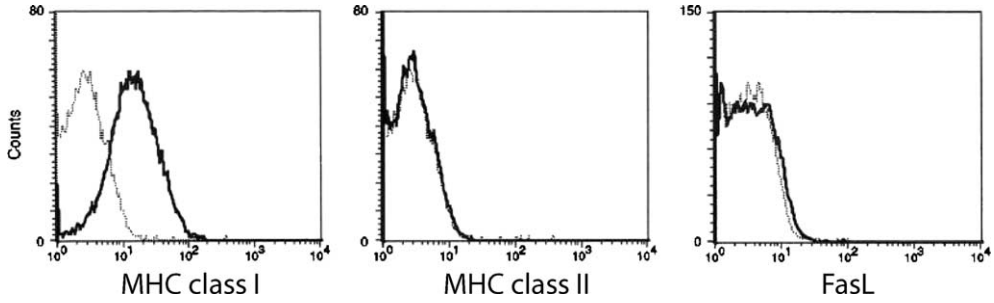

Fig. 4. Cultured AHPCs were examined for expression of MHC molecules and Fas Ligand (FasL) by flow cytometry. Low level class I expression was evident whereas labeling for class II (Ia) and FasL was similar to isotype controls. Bold line = antigen, fine broken line = isotype.

low compared to expression following stimulation of with IFN-γ (Fig. 5). No class II expression was detected at baseline. Both class I and class II antigens were strongly expressed by Fischer 344 spleen cells used as a positive control.

To further evaluate the effect of differentiation on factors potentially involved in regulating immunogenicity, AHPCs were cultured in FBS and evaluated for expression of MHC antigens after 6 days and 12 days. Expression of MHC antigens was not increased by this treatment at either time point (data not shown). Low level expression of class I was again present, although appearing to diminish with time of treatment. Class II was not expressed at either time point.

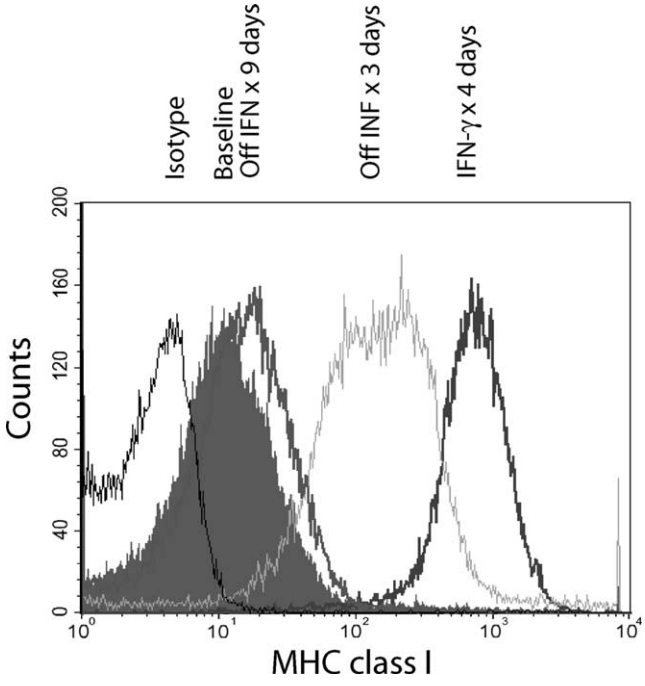

Fig. 5. Dynamic regulation of MHC class I expression by rat IFN-γ. Flow cytometric analysis shows marked induction of class I antigen after 4 days of treatment (peak at far right) as compared to baseline (solid peak) or isotype control (peak at far left). Withdrawal of IFN-γ resulted in progressive loss of class I (middle peaks) back to baseline levels.

Fas Ligand (CD95 Ligand) is an important immunoregulatory molecule with the ability to protect cells by inducing apoptosis in lymphocytes. Flow cytometric analysis did not detect the presence of FasL antigen on the surface of cultured AHPCs (Fig. 4), although this antigen was readily confirmed on the surface of mononuclear cells used as positive controls. This finding is consistent with the inability of AHPCs to inhibit lymphocyte proliferation under the conditions used in this study.

While unstimulated AHPCs expressed low levels of MHC class I, the addition of IFN-γ to cultured AHPCs resulted in marked up-regulation of this antigen by treatment day 4 (Fig. 5). Subsequent removal of IFN-γ resulted in progressive decreases of class I antigen expression, returning to baseline levels within 9 days of withdrawal (Fig. 5). Treatment of AHPCs with IFN-γ resulted in low, but detectable, expression of MHC class II (data not shown).

3.5. Cytokine production by AHPCs

Using rat-specific primers, RT-PCR was used to further investigate cytokine expression by AHPCs. Evidence was obtained for expression of TGF-β1 by the neural progenitor cells (Fig. 6). As part of this analysis, additional evidence was obtained indicating little or no baseline expression of IFN-γ, IL-2, IL-4, or IL-10 by these cells.

3.6. AHPCs do not inhibit PBMC proliferation

One potential explanation for the absence of PBMC proliferation in response to xenogeneic AHPCs is that

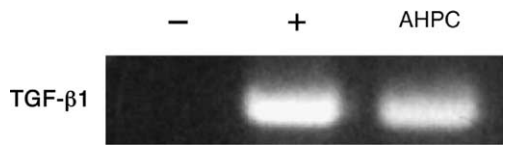

Fig. 6. RT-PCR performed on cultured AHPCs was positive for TGF-β1 gene expression (−lane = negative control, +lane = positive control).

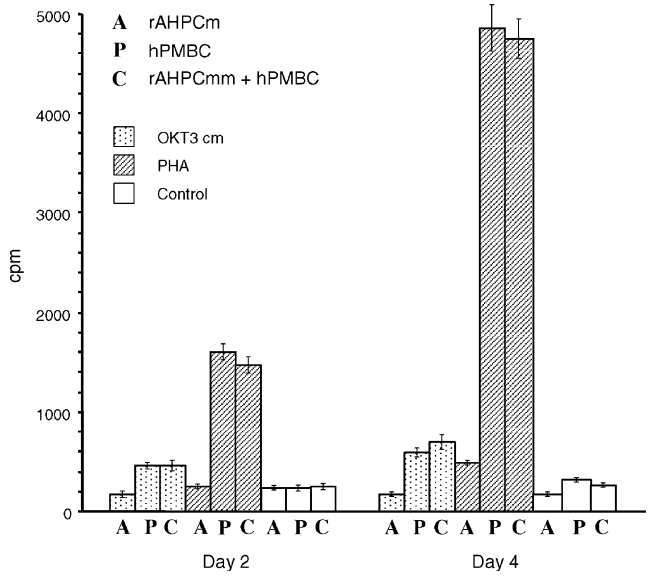

Fig. 7. PBMC stimulation study. To investigate whether AHPCs exert an inhibitory effect on PBMC proliferation, mitomycin-treated AHPCs were co-cultured with PBMCs in the presence of OKT3-conditioned supernatant or PHA. Both mitogens induced PBMC proliferation at both the 2 and 4 day time points. The addition of AHPCs did not impede this proliferation at either time point, for either mitogen. [^{3}H]TdR added 24 h prior to harvest.

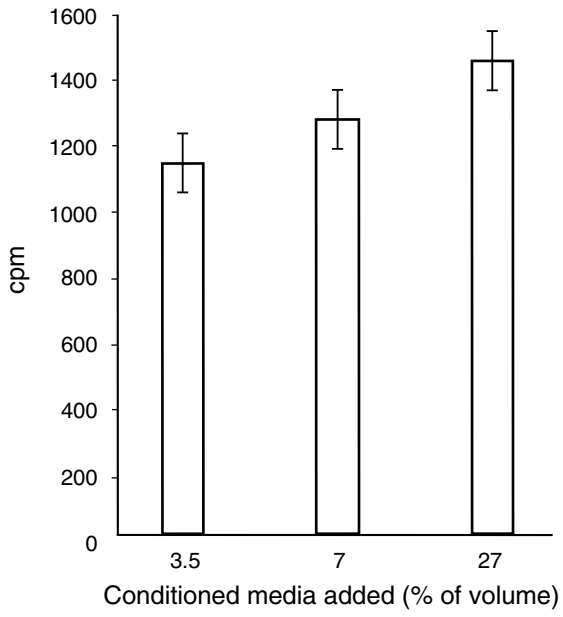

Fig. 8. Growth factor effect. Untreated AHPCs were cultured for 5 days in the presence of cell-free PBMC-conditioned supernatant, added at three different concentrations. Proliferation in the presence of conditioned supernatant was dose-dependent (positive linear trend, $P = 0.009$). [^{3}H]TdR was added on day 1. Mean, SEM.

the neural progenitor cells actively inhibit lymphocyte proliferation. To examine this possibility, experiments were performed under conditions in which lymphocyte proliferation was stimulated. The effect of combining AHPCs with proliferating PBMCs was evaluated at 2 day and 4 day time points. PBMCs exhibited marked proliferation in response to both OKT3 cell-conditioned supernatant and PHA, however, the presence of co-cultured AHPCs did not effect PBMC proliferation induced by either mitogen at either time point (Fig. 7).

3.7. PBMC-conditioned medium promotes proliferation of AHPCs

Because initial co-culture experiments indicated that PBMCs (and spleen cells) provide a proliferative stimulus to AHPCs, an additional experiment was performed to determine whether this effect could be reproduced using PBMC-conditioned supernatants alone. Cultured AHPCs were supplemented with 3.6, 7, or 27% PBMC-conditioned media by volume. [^{3}H]TdR was added on day 1 and samples were harvested on day 5 (Fig. 8). Increasing concentration of conditioned media was associated with increased [^{3}H]TdR uptake by AHPCs. This response was dose-dependent, consistent with the presence of a soluble factor (positive linear trend, $P = 0.009$).

4. Discussion

This study shows that a clonally-derived neural progenitor cell line exhibits non-immunogenic behavior when co-cultured with xenogeneic mononuclear leukocytes. The mechanism underlying this behavior does not necessitate active suppression of T cell proliferation, but rather, appears to result from evasion of immune recognition associated with baseline down-regulation of MHC antigen expression. Neural progenitor cells therefore appear to share at least some of the immunological characteristics of their progeny, the neurons and glia of the CNS. These findings have relevance for developmental biology as well as for work in neural stem cell transplantation.

It is well established that cell-mediated immunity is the major effector in transplant rejection. This process is initiated when host CD4+ T cells recognize foreign class II MHC molecules on the donor cells. Activated CD4+ T cells then provide help to host CD8+ T cells which in turn recognize the donor cells by way of foreign class I antigens and destroy them. Within this context, the absence of class II MHC expression by AHPCs, as shown in this study, would tend to protect them from immunological attack via elimination of the initiating stimulus, thereby providing an explanation for the lack of proliferation by co-cultured xenogeneic PBMCs seen here.

Another way in which CD4+ T cells can mediate the destruction of foreign cells is by activating macrophages

through the release of lymphokines. Again, this mechanism is dependent upon the recognition of foreign class II antigen by CD4+ T cells. Such a pathway does not appear to be activated under the in vitro conditions used here, given both the lack of class II expression by AHPCs and the absence of elevated IFN-γ in co-culture supernatants.

In addition to the direct rejection pathway, an indirect pathway has been described (Sayegh & Carpenter, 1996). This mechanism does not require the expression of class II MHC molecules by the foreign cells, but rather, the presence of free foreign antigen. These antigens are in turn presented to host CD4+ T cells in the context of class II MHC by host APCs, followed by induction of cytotoxic activity against the donor cells. Whereas a high proportion of lymphocytes respond to foreign class II MHC antigen directly without the need for inclusion of an additional foreign peptide fragment, the indirect pathway requires specific recognition of the foreign antigen being presented. The absence of PBMC proliferation in mixed co-cultures indicates that this indirect pathway was not evoked under the conditions used here. The specific recognition of rat antigens by human PBMCs would be unlikely in co-cultures because of the limited diversity of lymphocytes, and thus specific TCR idiotypes, within a given well. Such recognition might be possible in vivo, depending in part on individual history of exposure.

One specific antigen that deserves comment is the MHC class I molecule. It is clear from our flow cytometric data that AHPCs do express class I antigen, albeit at relatively low levels. This result is consistent with the generally low expression of MHC class I molecules by astrocytes and other resident cells of the CNS. It is equally clear that expression of class I antigen did not elicit proliferation of xenogeneic or allogeneic immune effector cells in vitro, likely for reasons cited above. Nevertheless, as we have shown, expression of class I and to some extent class II can be up-regulated by IFN-γ. The substantial increase in class I expression seen following exposure to this cytokine is consistent with the appropriate regulation of transplantation antigens by AHPCs, as is the return to baseline levels following withdrawal. This dynamic response to a pro-inflammatory mediator may in part explain the activation of neural progenitor cells by CNS injury, a crucial and poorly understood aspect of stem cell-mediated repair.

Another specific antigen of particular interest is GFP. Again, we saw no evidence of immune effector cell proliferation, indicating that expression of the enhanced version of this jellyfish protein by AHPCs did not result in antigen-mediated activation in vitro. Given the frequent use of this molecular labeling technique in transplantation studies, the immunological consequences of GFP expression are worthy of comprehensive in vivo characterization.

There are a number of additional pathways that can result in the immunological destruction of grafted cells. These include the recognition of minor transplantation antigens, antibody-associated rejection, as well as the activation of NK and γδ T cells. None of these various mechanisms would appear to be relevant to the results presented here. NK cells could potentially pose a threat to cells in which viral infection is used to insert a foreign gene. The theoretical relevance of γδ T cells lies in their ability to destroy cells in an MHC-unrestricted manner. Because γδ T cells appear to be localized to the external epithelial surfaces of the body, it seems unlikely that this interaction would pertain to foreseeable neural transplant scenarios.

A major consideration in all stem and progenitor cell transplantation is the generation of differentiated progeny following transfer to the host. The antigenic properties of these progeny may differ from those of their less differentiated parents, and their immunogenic properties may differ as well. Exposure of AHPCs to FBS is sufficient to induce the expression of neuronal, and especially glial, markers (Gage et al., 1995; Ray et al., 1993), yet insufficient to evoke a Th1 response from co-cultured xenogeneic PBMCs. Neither was there an associated increase in MHC expression by serum-treated AHPCs. This is consistent with the decreased MHC expression characteristic of mature CNS cells under normal conditions (Rall, 1998).

In addition to avoiding detection via decreased MHC antigen expression, AHPCs might actively suppress T cells, either through the secretion of factors or the expression of surface molecules. For instance, this study shows that AHPCs express TGF-β1, a factor with known lymphosuppressive activity. Interestingly, no inhibition of mitogenically-induced proliferation of co-cultured PBMCs was seen, either in the presence of anti-CD3 activity or PHA. The significance of TGF-β1 expression by neural progenitor cells awaits clarification, but might serve an autocrine role as a suppressor of MHC expression. The lack of FasL expression by AHPCs also argues against the alternative hypothesis of neural progenitor-mediated lympholytic activity.

Technical points worthy of mention include the use of serum- and bFGF-free conditions so as to moderate between the opposing tendencies of differentiation and proliferation, respectively. While not optimal for either co-culture cell type, this compromise provided a consistent baseline for comparison between experimental conditions. Although FBS is an effective differentiating agent, there can be non-specific effects, particularly at late time points. More specific agents such as CNTF, or retinoic acid and forskolin, might be useful to promote the differentiation of selected cell types. A retrovirally transfected cell line was used, largely because this same cell line is being used in parallel in vivo work (Young et al., 2000). Since no immunogenic response was elicited

by transfected AHPCs, this potential complication does not appear to have been involved here.

There are also a number of broader points of interest. In particular, the results of this in vitro study do not rule out the possibility that in vivo conditions could induce more immunogenic phenotypes, either in the progenitor cells or their progeny. For instance, pro-inflammatory conditions are associated with elevated levels of IFN-γ and TNF-α and up-regulation of MHC expression by neurons and glia (Neumann, Schmidt, Cavalie, Jenne, & Wekerle, 1997). Traditionally grouped among the last, microglia can more specifically described as the local APCs of the CNS. It is now widely accepted that microglia are of bone marrow origin and migrate into the CNS secondarily (Cuadros & Navascues, 1998). Nevertheless, the possibility that such cells can in some instances be of neuroepithelial origin remains difficult to rule out (Bjornson et al., 1999). This is important when considering whether grafted NSCs have the potential to differentiate into MHC class II-expressing "passenger" APCs and thereby facilitate their own rejection. In that regard, we have grafted neural progenitor cells from Fischer 344 rats into the diseased CNS of RCS rats and found sustained survival of allogeneic cells without obvious signs of rejection (Young et al., 2000). In that study, AHPCs appeared to respond in a tropic manner to the degenerative microenvironment. Analogous findings have also been reported in other models using NSC transplantation (Bjornson et al., 1999; Brustle et al., 1998). Pro-inflammatory cytokines are obvious candidates for such injury cues and might play a role in directing stem cell migration and differentiation in response to cell loss. The regional and temporal variations in cytokine and MHC antigen expression in the developing and mature CNS suggest a complex interplay of immunological and neural signaling (Huh et al., 2000; Neumann et al., 1997), the full scope of which remains to be defined.

The immunological characterization of neural stem cells is now beginning. The present study shows that a neural progenitor line, clonally-derived from the hippocampal formation of adult rats, exhibits non-immunogenic behavior in vitro. One factor that appears to contribute to this behavior is low level class I and no detectable class II (Ia) MHC antigens. In this respect, neural progenitors, at least those found in the hippocampal formation of mature rats, resemble the neurons and glia to which they give rise. Immunologically, neural progenitor lines therefore provide a major advantage over neural tissue grafts in that they are free of immunogenic cell types such as passenger leukocytes and endothelial cells. Furthermore, neural progenitors do not appear to differentiate into immunogenic cell types under a range of in vitro or in vivo conditions. Additional studies are indicated to determine the extent to which the results of this study generalize to neural progenitor lines derived at different developmental time points, from different CNS regions, and from other mammalian species.

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