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Catecholaminergic neurons result from intracerebral implantation of embryonal carcinoma cells

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ABSTRACT **A replication-defective retrovirus was used to introduce the marker gene *nlsLacZ* into the murine embryonal carcinoma (EC) cell line PCC7-S-aza-R-1009. Undifferentiated EC cells were implanted into the central nervous system of adult rats. One month later, the grafted cells continued to express the *nlsLacZ* gene. Immunohistochemical analysis demonstrated the presence of EC-derived neurons. These neurons were capable of expressing tyrosine hydroxylase and extended neurites into the host parenchyma. EC-derived glial cells could not be detected. There was no evidence of tumorigenicity. These results demonstrate the utility of EC cells for introduction of exogenous gene products into the central nervous system in experimental models of gene therapy.**

Progress has been made toward treatment of a variety of diseases by genetic means. The most common approach taken involves introduction of genes into either cells taken from the body or established cell lines. The genetically modified cells are then implanted back into the body where the gene product will complement a deficiency (1). This strategy has recently been applied to the central nervous system (CNS) as a possible treatment for a number of neurodegenerative disorders including Alzheimer and Parkinson diseases (2–6). Cell lines potentially useful as vehicles for delivering exogenous gene products to the CNS ideally should meet certain criteria: (*i*) nontumorigenicity after implantation, (*ii*) ready incorporation and expression of foreign genes, and (*iii*) integration into the host brain parenchyma to permit increased diffusion of the introduced gene product.

A variety of genetically modified cell lines have been transplanted into the CNS to serve as delivery systems for the transgene of interest. These include cell lines derived from tumors such as PC12, ATt20, and neuroblastomas (7–10); nontransformed immortalized cell lines such as the fibroblastic NIH 3T3 and Rat 208F (11, 12); and primary fibroblasts (6). Embryonic cells taken directly from the developing fetal CNS have also been used (13, 14). An alternative possibility would be the use of immortalized neural cell lines. Such cell lines have been isolated from neural tissues directly or through the use of immortalizing genes (15–17).

Another alternative is the use of embryonal carcinoma (EC) cells. EC cell lines possess many of the same characteristics as stem cells from the embryo and have therefore been studied as models of early embryonic development. Specifically, these cell lines possess the ability to differentiate into a variety of cell types either *in vitro* under controlled conditions or *in vivo*. Some EC cell lines also possess the ability to differentiate into neural phenotypes (18). To assess the possible use of EC cell lines as a vehicle to deliver neurotrophic factors and/or neurotransmitters to the adult brain, we chose the EC cell line PCC7-S-aza-R-1009 (1009 cells). Previous studies have shown that 1009 cells possess an apparently normal diploid karyotype (19). When induced to differentiate *in vitro* by aggregate formation or with retinoic acid, 1009 cells preferentially yield neural derivatives—neurons and glia—as confirmed by intermediate filament expression (20). The neuronal cells possess neurites, which resemble those observed in primary neuronal cultures and are capable of producing neurotransmitters (21).

A replication-defective retrovirus (dRRV) vector was used to introduce the marker gene *nlsLacZ* into 1009 cells. Populations that were enriched for marker gene expression by fluorescence-activated cell sorter (FACS) selection were stereotaxically placed into the adult rat brain. These implanted cells continued to express the marker gene 5 weeks later. Immunohistochemical analyses revealed that the cells had differentiated *in vivo* into neurons but not glial cell types. The neurons also exhibited reactivity with anti-tyrosine hydroxylase (TH) antibodies. No evidence of malignant tumor formation was observed.

METHODS AND MATERIALS

Cell Culture and Retrovirus Infection. All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. 1009 cells are derived from a spontaneous testicular teratocarcinoma arising in a recombinant inbred mouse strain (129 × B6) (19). The 1009 cells used for this study were passaged at subconfluence (18).

The dRRV-producing cell line PA12ψ-221C3 has been described (22). The dRRV obtained from this cell line contains the *nlsLacZ* gene under the control of the simian virus 40 (SV40) early region promoter. The virus present in the culture medium was concentrated by centrifugation and then used to infect 1009 cells in the presence of Polybrene (5 mg/ml) as described (22).

FACS. The *nlsLacZ*-expressing 1009 cells were selected and subcloned by FACS using fluorescein digalactosidase as a vital stain for β-galactosidase activity (23). One week later, clones were tested for *nlsLacZ* by β-galactosidase histochemistry using 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal; Sigma) as described (22). The cells were fixed

Abbreviations: CNS, central nervous system; EC, embryonal carcinoma; dRRV, replication-defective retrovirus; FACS, fluorescence-activated cell sorter; TH, tyrosine hydroxylase; SV40, simian virus 40; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside; NGS, normal goat serum; NSE, neuron-specific enolase; GFAP, glial fibrillary acidic protein.

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with 4% formaldehyde/0.2% glutaraldehyde in phosphate-buffered saline (PBS) (pH 7.5). Positive clones were maintained in culture and periodically tested with X-Gal to determine the stability of expression. The most stable of these clones, designated A6, was used for most of this study.

***In Vitro* Immunohistochemical Labeling.** *In vitro* differentiation with retinoic acid and cAMP was performed as described (18). Six days after the start of retinoic acid treatment, the cells were fixed with 4% paraformaldehyde/0.2% picric acid in PBS (pH 7.5), rinsed with PBS, and then stained with X-Gal (22) overnight at 37°C. After rinsing with PBS, nonspecific antibody binding sites were blocked by a 30-min incubation with 3% normal goat serum (NGS)/0.3% Triton X-100 in PBS. The blocking solution was removed and the cells were incubated at 4°C overnight with the primary antibody in 1% NGS/0.3% Triton X-100 in PBS. Cells were incubated with either rabbit polyclonal antiserum to neuron-specific enolase (NSE; 1:800; ref. 24), glial fibrillary acidic protein (GFAP; Dakopatts; 1:300; ref. 29), or TH (1:2000; Institut Jacques Boy S.A.). The labeling was visualized by using the Vectastain ABC kit (Vector Laboratories) followed by reaction with 0.025% 3,3'-diaminobenzidine tetrahydrochloride (Sigma) and 0.013% H_2O_2 in 0.05 M Tris buffer.

Lesioning and Grafting. Undifferentiated β-galactosidase-positive 1009 cells (2×10^5 to 10^6) were stereotaxically placed into the region of the ventrobasal thalamic complex (VB) in adult rats in which the VB had previously been excitotoxically depleted of neurons by kainic acid (25). A total of 23 adult female Sprague–Dawley albino rats (225–250 g) underwent the lesioning/grafting procedure. The rats were anesthetized with chloral hydrate (i.p.; 400 mg/kg) during all surgical procedures. A 0.15-μl injection of the excitotoxin kainic acid (0.5% in saline) was stereotaxically delivered over a period of 15 min to the diencephalon. Either 2 or 4 weeks after lesioning 2–3 μl of a suspension of 1009 A6 cells (100,000–250,000 cells per μl of PBS or DMEM) were stereotaxically placed into the lesioned area. Daily injections of cyclosporin A (i.p.; 16.5 mg/kg) were given from the day before grafting and continued until the day before the rats were sacrificed. Six of the 23 rats did not receive cyclosporin.

Histology and Immunohistology. Four to 5 weeks after grafting the rats were overdosed with sodium pentobarbitone (64.8 mg) and then perfused transcardially with warm PBS containing heparin (3 units/ml) followed by 4% paraformaldehyde/0.2% picric acid in 0.1 M phosphate buffer (pH 7.5). The brains were removed, postfixed for 1–2 hr, transferred to 30% (wt/vol) phosphate-buffered sucrose, and kept overnight at 4°C. The brains were frozen and 30-μm sections were made using a freezing microtome. Floating sections were then incubated in X-Gal (400 mg/ml X-Gal/4 mM potassium ferricyanide/4 mM potassium ferrocyanide/4 mM $MgCl_2$ in PBS at pH 7.5) at 34°C overnight. Some of the adjacent sections were then counterstained with either neutral red or cresyl violet. The other sections were stained immunohistochemically for either NSE or GFAP as follows. The floating sections were rinsed three times with PBS followed by a 30-min incubation at room temperature in 1% H_2O_2 to reduce background peroxidase activity. After rinsing with PBS, nonspecific antibody binding sites were blocked by a 30-min incubation with 3% NGS/0.3% Triton X-100 in PBS. The blocking solution was removed and the sections were incubated at 4°C overnight with the primary antibody in 1% NGS/0.3% Triton X-100 in PBS. Alternate sections were incubated with rabbit polyclonal antibodies to TH (1:2000), NSE (1:800), or GFAP (1:300). After thorough rinsing with buffer, the labeling was visualized by using the Vectastain ABC kit followed by reaction with 0.025% 3,3'-diaminobenzidine tetrahydrochloride (Sigma) and 0.013% H_2O_2 in 0.05 M Tris buffer. The sections were then mounted on gelatinized slides and cleared in increasing concentrations of ethanol followed by toluene. Coverslips were mounted with Permount.

RESULTS

A dRRV (MMoLVSVnlsLacZ) vector was used to introduce a marker gene, *nlsLacZ*, under control of the SV40 early gene promoter, into 1009 cells. The *nlsLacZ* gene consists of the SV40 nuclear localizing sequence (*nls*) fused to the *Escherichia coli LacZ* coding sequence. The gene product, β-galactosidase, retains its activity but is now localized to the nucleus by the peptide coded from the SV40 *nls* (22). As eukaryotic β-galactosidase is localized to the cytoplasm (in lysosomes), specific histological staining of grafted 1009 cells, which have been previously infected by this retrovirus, can be distinguished from any nonspecifically stained host cells (i.e., macrophages, endothelial cells) by the intracellular location of the histochemical stain.

Initially, <1% of the dRRV *nlsLacZ*-infected 1009 cells expressed detectable levels of β-galactosidase after infection. Enriched populations of β-galactosidase-positive cells were obtained by FACS selection using fluorescein digalactosidase to label living cells (23). Unfortunately, these populations did not stably express the *nlsLacZ* gene *in vitro*. However, a clonal population of stable β-galactosidase-positive cells was obtained by FACS selection as described above. This clone, designated A6, continued to express the *nlsLacZ* gene in a majority of the cells in the population after 2 months in culture (Fig. 1).

In vitro differentiation of 1009 cells with retinoic acid and cAMP normally yields neuronal and glial phenotypes (18, 20, 21). *In vitro* differentiation of the *nlsLacZ*-expressing clone A6 cells with retinoic acid and cAMP yielded neuronal and glial phenotypes as demonstrated morphologically and by NSE, TH, and GFAP immunostaining (Fig. 2). In addition to our controls, in which cells were incubated with serum-containing buffer in the place of primary antibody, the specificity of immunostaining is clearly demonstrated by the unstained cells in Fig. 2. Our *in vitro* experiments have thus demonstrated expression of an exogenous marker gene in 1009 cells both before and after differentiation and, further-

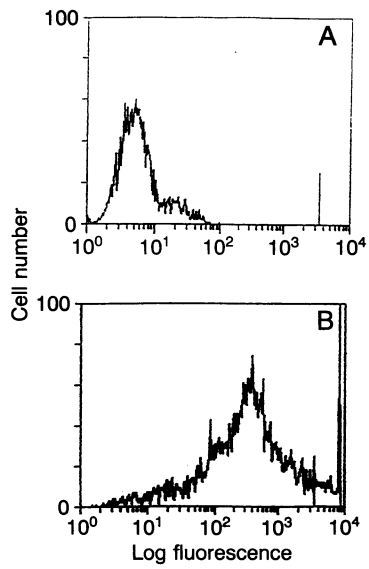

FIG. 1. FACS-derived histograms of fluorescein digalactosidase-stained 1009 cells. (*A*) Uninfected 1009 cells (as a control). (*B*) Clone A6. FACS analysis performed 1 month in culture after the initial FACS cloning. *x* axes, logarithm of fluorescent product; *y* axes, cell number. 1009 cells were processed as described.

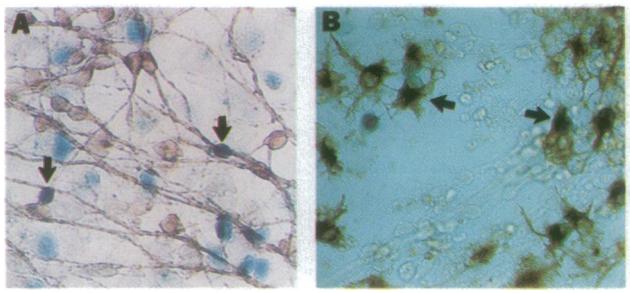

FIG. 2. *In vitro* immunostaining of differentiated *nlsLacZ*-expressing 1009 cells. (*A*) Anti-TH antibody. Arrows indicate cells that possess both a nucleus exhibiting β-galactosidase activity and cytoplasmic anti-TH immunostaining. All of the cells with a neuronal morphology are NSE immunoreactive and most are TH immunoreactive. The β-galactosidase-positive nuclei of cells that are not TH immunoreactive or NSE immunoreactive (data not shown) can be seen. These cells are morphologically flat and do not possess neurites. It should be noted that in the majority of the NSE-immunoreactive or TH-immunoreactive neurons localization of the *nlsLacZ* gene product has changed from its usual perinuclear location (22) to the cytoplasm. Electron microscopy has indicated that the gene product is now associated with the endoplasmic reticulum (B.E.W., unpublished results). This shift is not seen in grafted 1009 clone A6, which have differentiated into neurons *in vivo*. Thus, one is left to speculate that this shift is an *in vitro* artifact resulting from intracellular changes, possibly caused by the lack of a three-dimensional matrix during differentiation. (*B*) Anti-GFAP. Arrows indicate cells that possess both a nucleus exhibiting β-galactosidase activity and cytoplasmic anti-GFAP immunostaining. Unlabeled neurites can also be seen, further demonstrating the specificity of the antibody. Most of the GFAP-immunoreactive cells exhibited nuclear β-galactosidase staining. Data are representative of those obtained in at least 10 separate experiments.

more, that the expression of this gene does not influence the phenotypic commitment of the differentiating 1009 cells.

In vivo, 5 weeks after stereotactic implantation into the neuron-depleted ventrobasal thalamic complex in adult rats, 1009 cells exhibit nuclear-specific coloration characteristic of *nlsLacZ*-expressing cells after histochemical staining of sections with X-Gal. Cells exhibiting nuclear labeling were found only in the graft. The intracellular labeling varied from small points of stain to an area corresponding to that of a nucleus. In some of the sections there was also some nonspecific, diffuse, cytoplasmic staining observed in a few of the cells within the graft. This coloration has been previously shown to be associated with macrophages (26). In addition, there was nonspecific cytoplasmic staining evident in the endothelial cells lining the vessels throughout the brain and an especially strong cytoplasmic labeling of the choroid plexus. There was no labeling, nuclear or cytoplasmic, of neurons or glia outside of the graft.

Grafts survived in <20% (1/6) of the rats that did not receive cyclosporin, whereas a survival rate of ≈90% (15/17) was noted for the rats receiving cyclosporin. The size and cell density of the grafts were variable, from an estimated few hundred cells, many of which could be distinguished as separate cells, to very large grafts containing an inestimable number of very densely packed cells. In grafts of low cell density, it was easy to see that many of the cells possessed neuronal features similar to neurons in the adult brain (Fig. 3*A*). None of the grafts expanded beyond the lesioned area into which the cells had been implanted and infrequently comprised the total area of the lesion. A glia limitans was not observed. This suggests that the lack of expansion is not due to containment by a glial band. The grafts are generally well vascularized by vessels that vary in size from small to very large vessels not normally seen in the host parenchyma.

Since undifferentiated 1009 cells *in vitro* possess relatively small nuclei compared to the large labeled nuclei observed in

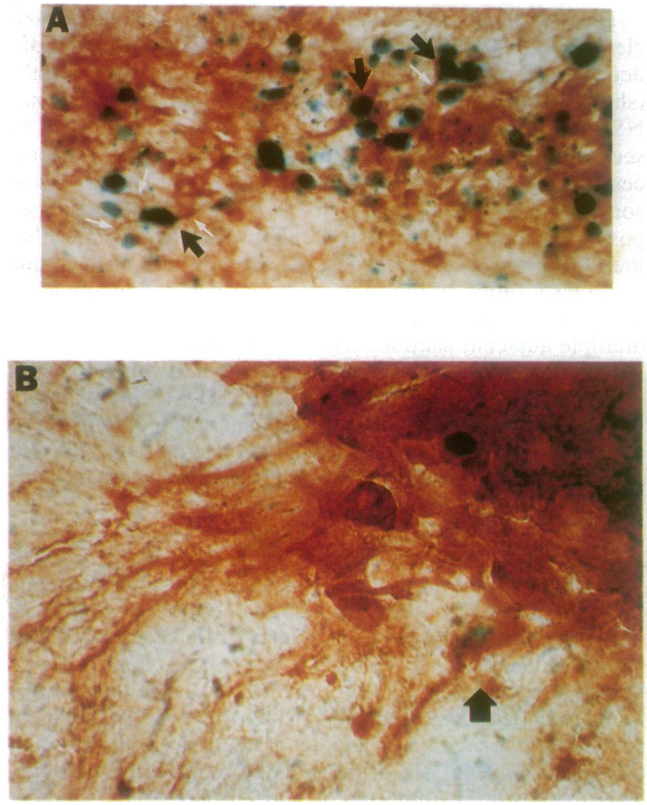

FIG. 3. Double labeling of intracerebrally grafted 1009 cells with X-Gal and anti-NSE antibodies. (*A*) Anti-NSE antibody. Section (30 μm thick) through a graft of relatively low cell density (rat 17). A number of cells that possess both a nucleus exhibiting β-galactosidase activity and cytoplasmic NSE immunostaining can be seen (black arrows). For some of these cells NSE-immunoreactive neurites can also be observed (white arrows). (×200.) (*B*) Anti-NSE antibody. View of the periphery of a graft of relatively high cell density (rat 14). Mass of the graft is on the upper right. NSE-immunoreactive neurites extend out of the graft into the host brain parenchyma. A 1009 cell-derived neuron exhibiting nuclear β-galactosidase activity and cytoplasmic NSE immunostaining, including a neurite, is indicated by the arrow. (×500.)

the graft, the morphological characteristics of the cells in the grafts suggested that the grafted 1009 cells had differentiated. To determine the resulting phenotypes, we took advantage of the nuclear β-galactosidase labeling to perform a double labeling with antibodies to either GFAP, NSE, or TH. Adjacent sections were reacted with antibodies to NSE, TH, and GFAP, followed by the appropriate secondary antibody as described above. As shown in Fig. 3, there was very strong labeling of the grafted cells with the anti-NSE antibody, indicating that 1009 cells had differentiated along neuronal lineages. This strong NSE immunoreactivity sharply contrasts with the surrounding tissue, which was previously depleted of host neurons by kainic acid and therefore displays no NSE immunoreactivity. Due to the high cell density in the majority of grafts, it was not always possible to distinguish individual cells, although a few individual cells could be seen in parts of the grafts having low cell density (Fig. 3*A*) and at the periphery (Fig. 3*B*). These cells possessed neuritic processes that extended into the surrounding host parenchyma. This observation was supported by evidence from 1009 cells prelabeled with horseradish peroxidase (HRP). Observation at the light and electron microscopic level 9 days after implantation revealed that HRP-containing cell processes had penetrated into the surrounding host parenchyma (B.E.W. and M.P., unpublished results).

The labeling with anti-GFAP antibody resulted in no clearly double-labeled GFAP-reactive glia in the graft. Indeed, the grafts were relatively clear of GFAP-reactive cells (shown in Fig. 4*A*), as compared to the labeling with anti-NSE antibody. In this representative view, the graft can be seen as an area containing many GFAP-reactive cell processes but apparently few GFAP-reactive cell bodies. While some of these cells appear to possess a β-galactosidase-positive nucleus, adjustment of the focal plane indicates that the GFAP-reactive cell processes are not part of the same cell. Observation of the sections under higher magnification (Fig. 4*B*) support this conclusion as did observation of multiple adjacent sections from several different rats.

Labeling with anti-TH antibody revealed that the 1009 cells in the graft contained TH (Fig. 5). Strong TH immunoreactivity was observed in the cell soma and primary neurites of many cells, but the high cell density in the graft did not permit definition of a possible subset of TH-expressing cells.

DISCUSSION

When implanted into the testes or under the kidney capsule of adult mice, 1009 cells form teratocarcinomas containing derivatives of all three primary germ cell layers (27). Our results indicate that 1009 cells will give rise to neurons upon being grafted intracerebrally. The cell density of the neurons found in the grafts as compared to the cell density of adjacent sections stained with cresyl violet or neutral red suggests that the majority, if not all, of the cells in the graft are neurons. This conclusion is supported by anti-GFAP immunostaining. Observation of multiple sections from several rats indicated that 1009 cell-derived GFAP-reactive glial cells could not be found in any of the grafts. Thus, it can be stated that while *in vitro* differentiation of 1009 cells results in neurons and glia, the implantation of 1009 cells into the adult rat thalamus primarily restricts their development to neuronal lineages. This could be the result of the presence of factors (i.e., growth factors, trophic factors, extracellular matrix proteins, etc.) in the adult brain that influence the choice of developmental lineage in differentiating cells (instructive lineage). Alternatively, the implanted 1009 cells could start to differentiate into a variety of cell types, including glia, followed by the survival of only the neurons (selective lineage).

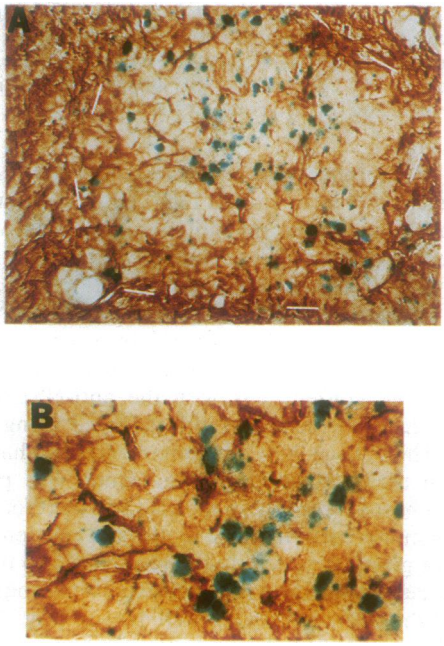

FIG. 4. Immunolabeling of intracerebrally grafted 1009 cells with X-Gal and anti-GFAP antibodies. (*A*) Anti-GFAP. Section (30 μm thick) through a graft of high cell density (third section adjacent to the one shown in 4*B*) (rat 14). White lines indicate approximate border of the graft. (×125.) (*B*) Anti-GFAP. Magnification of the center of the field in *A*. GFAP-immunoreactive fibers appear to pass over or under the X-Gal-staining nuclei. Observation while adjusting the focal plane supports this conclusion. (×500.)

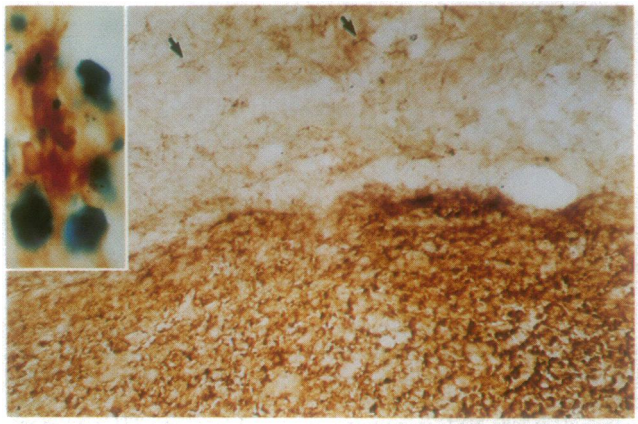

FIG. 5. Immunolabeling of intracerebrally grafted 1009 cells with anti-TH antibody. Graft can be seen in the lower portion as a region exhibiting TH immunoreactivity. TH-immunoreactive fibers of host origin traverse the host parenchyma (arrows). (×80.) (*Inset*) Another graft double labeled with X-Gal and anti-TH antibodies at a higher magnification. (×50.)

One of the criteria for evaluating the potential usefulness of a particular cell line as a vehicle for gene delivery is integration (or, at least, lack of exclusion) into the host brain parenchyma. Our observations of the grafts of 1009 cells revealed no strong evidence of a glial band. Indeed, our results indicate that the β-galactosidase-positive 1009 cell-derived neurons are not isolated from the host brain parenchyma. Cell processes can be observed at the periphery of many of the grafts extending into the surrounding host parenchyma. This increased integration into host tissue by the neurites should permit increased diffusion of any introduced gene product and therefore augment its effectiveness. It is not yet known whether the neurites from 1009 cell-derived neurons can form synapses with host cells.

Neuroblastoma and glioma cell lines have been used in grafting to the CNS. While capable of integrating into the host parenchyma these cell lines realize their tumorigenic potential after implantation. Although we have not extensively studied the tumorigenicity of 1009 cells after implantation into the CNS, the results of our study suggest that the grafted 1009 cells are not highly tumorigenic. One month after implantation, the size of the grafts is relatively small—similar in size to grafts of embryonic tissue at identical times postimplantation (25). Also, no expansion of the graft outside the lesioned area was observed. A glia limitans was not present in any of the grafted rat brains, indicating that this was not the reason for the observed lack of expansion of the grafted cells. Furthermore, the use of cyclosporin lessens the probability that our failure to observe rapidly expanding tumors is due to host immune response. Preliminary results obtained from autoradiographic analyses using [^{3}H]thymidine to label proliferating cells indicate that <10% of the cells in the region comprising the implanted cell mass continued to divide at 9 days and 4 weeks postimplantation (unpublished results). These cells were distributed in discrete clusters throughout the graft. It is interesting to note that for the one brain that was simultaneously stained with X-Gal none of the β-galactosidase-expressing cells continued to divide. This result suggests that the dividing cells observed are the result of host gliosis, which is a commonly observed phenomenon in lesioning/grafting experiments (28). These observations do

not exclude the possibility of slow-growing tumorigenic cells. However, the absence of tumorigenicity remains a likely possibility.

In an effort to determine the capacity of 1009 cells to permit the ready incorporation and expression of foreign genes, we introduced the marker gene *nlsLacZ* into 1009 cells. The relatively stable expression of the *nlsLacZ* gene in a population of 1009 cells was achieved by cloning through the use of the FACS. *In vitro* differentiation of these cells resulted in expression of the *nlsLacZ* gene in roughly the same proportion of cells as seen prior to differentiation and in undifferentiated sibling cultures. Our results have also demonstrated continued expression of the retrovirally introduced *nlsLacZ* gene from the SV40 promotor to up to 5 weeks postimplantation. The utility of the *nlsLacZ* gene as a marker was demonstrated, as it permitted simultaneous use of immunocytochemical staining to identify and distinguish among groups of cells (i.e., neurons vs. glia; TH-reactive neurons). In addition, use of the *nlsLacZ* gene eliminates the requirement for macrophage-specific immunostaining to distinguish these macrophages from grafted cells.

TH immunoreactivity of these 1009 cell-derived neurons suggests that this EC cell line could be useful in experimental studies of Parkinson disease, as they could supply missing factors without being genetically modified beforehand. In summary, our results indicate that 1009 cells may be suited for use as a vehicle for delivery of various agents that intervene in the process of neurodegenerative disorders.

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