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Improvement of neurological deficits in 6-hydroxydopamine-lesioned rats after transplantation with allogeneic simian virus 40 large tumor antigen gene-induced immortalized dopamine cells

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ABSTRACT **The replacement of dopamine (DA) by DA neuron transplants in the treatment of advanced Parkinson disease (PD) is a rational approach. Because of limitations associated with fetal tissue transplants, a clone ($1RB_3AN_{27}$) of simian virus 40 large tumor antigen (LTa) gene-induced immortalized DA neurons were used in this study. These allogeneic immortalized dopamine neurons, when grafted into striata of normal rats, did not divide, did not form tumors, did not produce LTa, did not extend neurites to host neurons, and were not rejected, for as long as 13 months after transplantation. Grafted cells when recultured *in vitro* resumed cell proliferation and LTa production, suggesting the presence of a LTa gene-inhibiting factor in the brain. The grafting of undifferentiated and differentiated $1RB_3AN_{27}$ cells or differentiated murine neuroblastoma (NBP_2) cells into striata of 6-hydroxydopamine-lesioned rats (an animal model of PD) caused a time-dependent improvement in neurological deficits (reduction in the methamphetamine-induced turning rate). At 3 months after transplantation, 100% of the animals receiving differentiated $1RB_3AN_{27}$ cells, 63% of the animals receiving undifferentiated $1RB_3AN_{27}$ cells, 56% of the animals receiving differentiated NBP_2 cells, and 0% of the sham-transplanted animals showed improvements in neurological deficits. At 6 months after transplantation, there was a progressive increase in spontaneous recovery in sham-transplanted animals. These results suggest that immortalized DA neurons should be further studied for their potential use in transplant therapy in advanced PD patients.**

The replacement of dopamine (DA) by fetal mesencephalic tissues or DA-cell transplant appears to be a rational choice for the treatment of patients with advanced Parkinson disease (PD). Fetal mesencephalic tissues have been used extensively in animal models of PD (1–7) with various levels of improvement of neurological deficits. The use of such tissues in advanced PD cases also produced variable results (8–11). A double-blind study with placebo control to evaluate the efficacy of fetal tissue in the treatment of neurological symptoms in patients with advanced PD is in progress (C.R.F., unpublished work). Apart from the issue of efficacy of allogeneic fetal central nervous system tissue in neural transplants, the use of fetal tissue is limited by ethical, legal, tissue availability, survivability, and some inherent biological problems. The latter includes the presence of donor antigen-presenting cells that may induce rejection of allogeneic grafted tissues (12) and heterogenicity within the population of grafted cells. To overcome the above difficulties, the establishment of homogeneous populations of DA-producing neurons *in vitro*, free of antigen-presenting cells, could be invaluable. Clonal lines of transformed (tumorigenic) DA-producing neurons (13–16) are available and have been used in neural transplant studies (17, 18), but such cells produce tumors after transplantation; therefore, they may not be useful until 100% of them are terminally differentiated prior to grafting. Recently, we have reported the production of 100% terminally differentiated murine neuroblastoma (NBP_2) cells in culture (19); however, the efficacy of these cells in improving the neurological deficit in the rat model of PD is minimal at 30 days after transplantation (20). In an effort to establish another source of DA-producing cells, spontaneously or genetically engineered immortalized cells, which include tyrosine hydroxylase (TH)-producing fibroblasts (21), dopamine-producing simian virus 40 (SV40) large tumor antigen (LTa) gene-induced transformed cells at permissive temperature (22), and multipotent neural cells (23), are now available. The grafting of these cells in animal models of PD has produced variable improvements in neurological deficits when assayed within 3 months of transplant. Recently, we have established an immortalized clone of rat DA-producing neurons ($1RB_3AN_{27}$) by transfecting fetal mesencephalon cells with the plasmid vector pSV_3^{neo}, which carries the LTa gene from SV40 virus (24). When these cells or differentiated NBP_2 cells were grafted into the striata of 6-hydroxydopamine (6-OHDA) lesioned rats, the methamphetamine-induced turning rate was reduced in about 50% of transplanted animals at 30 days after transplantation; and there was no evidence of rejection of allogeneic cells nor tumor formation at this time. Because this observation period was considered too short for determination of the efficacy of transplanted cells in improving the neurological deficits in lesioned rats, we extended the period of observation to 6 months. In addition, we compared the relative efficacy of undifferentiated and differentiated $1RB_3AN_{27}$ cells, at improving the neurological deficits in the rat model of PD.

We report the following observations. (*i*) Grafting of allogeneic $1RB_3AN_{27}$ cells into striata of normal rats revealed no evidence of cell proliferation, LTa production, tumor formation, rejection, or axonal innervation at 1, 4, 8, and 13 months after transplantation. (*ii*) Grafted allogeneic $1RB_3AN_{27}$ cells, when recultured *in vitro*, resumed cell proliferation and LTa production. (*iii*) Grafting of differentiated $1RB_3AN_{27}$ cells was

Abbreviations: DA, dopamine; 6-OHDA, 6-hydroxydopamine; LTa, large tumor antigen; dbcAMP, dibutyl cAMP; DHEA, dehydroepiandrosterone; PD, Parkinson disease; TH, tyrosine hydroxylase; DAT, dopamine transporter protein; SV40, simian virus 40; RT–PCR, reverse transcriptase-coupled PCR.

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most effective in comparison to grafting of undifferentiated $1RB_3AN_{27}$ cells or differentiated murine NBP_2 cells in reducing amphetamine-induced turning rate in 6-OHDA lesioned rats at 3 and 6 months after grafting. However, at 6 months after transplantation, there was evidence of spontaneous recovery in sham-transplanted animals.

MATERIALS AND METHODS

Cell Culture. The clonal line of immortalized DA-producing neurons $1RB_3AN_{27}$, which has been established in our laboratory, was used (24). Cells were grown in RPMI medium 1640 containing 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) and were maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air. Mycoplasma-free cultures were used for all experiments. The doubling time of $1RB_3AN_{27}$ cells was about 26 h.

A murine neuroblastoma clone (NBP_2), which was developed in our laboratory (14), was also used in this study. Cells were grown in F12 medium containing 10% gamma-globulin-free newborn bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) and were maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air. The doubling time of NBP_2 cells was about 18 h.

Differentiating Agents. $1RB_3AN_{27}$ cells (0.5×10^6 cells) were plated on tissue culture dishes (100 mm) that were precoated with specialized substrate (25) for 4 h. Dibutyryl adenosine 3′,5′-cyclic monophosphate (dbcAMP) at 2.0 mM induces morphological and biochemical differentiation in some cells (20); however, the addition of dehydroepiandrosterone (DHEA) at 60 μg/ml enhanced the level of morphological differentiation in these cells 3 days after plating (K.N.P. and J.E.-P., unpublished observations). Therefore, these agents were used in combination to induce optimal differentiation in $1RB_3AN_{27}$ cells. Murine neuroblastoma (NBP_2) cells (0.5×10^6 cells) were plated on regular tissue culture dishes (100 mm). 4-(3-Butoxy-4-methoxy-benzyl)imidazolidin-2-one (R020–1724, 200 μg/ml), an inhibitor of cyclic nucleotide phosphodiesterase, plus a polar carotenoid, originally referred to as β-carotene (20 μg/ml), are known to induce terminal differentiation in NBP_2 cells (19); therefore, they were used in this study. Differentiating agents were added 24 h after plating, and growth medium and agents were changed at 2 days after treatment. Various biological assays including transplantation were performed at 3 days after treatment when differentiated functions were maximally expressed.

Assay of TH and Dopamine Transporter Protein (DAT) mRNA. $1RB_3AN_{27}$ cells (0.5×10^6 cells) were collected in a microcentrifuge tube. The cells were centrifuged and the supernatant was removed. An Oligotex direct mRNA isolation kit (Qiagen, Chatsworth, CA) was used to isolate total RNA from the cells. The RNA was quantified with a spectrophotometer to ensure that equal amounts of RNA were loaded into each reverse transcriptase-coupled PCR (RT–PCR) mixture. The isolated RNA was then stored at −20°C until it was needed.

The forward primer for TH was GGGGAGCTGAAGGCTTATGG (nucleotides 1161–1268) with the reverse for TH being GGACACAAAGTACACAGGCT (nucleotides 1316–1297). The forward primer for the DAT was TATGGCACAGAAGCACAATGTGCCCA (nucleotides 1169–1194) with the reverse for DAT being AGCCAGGACAATGCCAAGAGTGAAGA (nucleotides 1424–1399). The TH primers have been described (26). The reactions were carried out in a Perkin–Elmer GeneAmp PCR system model 9600. The reverse transcriptase reaction synthesized cDNA from 1 μg of total RNA by using 2.5 μM of random hexomers, 50 units of murine leukemia virus reverse transcriptase, 20 units of RNase inhibitor, 1 mM dGTP, 1 mM dATP, 1 mM dTTP, and 1 mM dCTP, 5 mM $MgCl_2$, and 1× PCR buffer II in a total volume of 20 μl (all reagents were from Perkin–Elmer). The mixture was incubated at room temperature for 10 min and was then placed in the thermocycler that was set at 42°C for 15 min, 99°C for 5 min, and 5°C for 5 min. After the reverse transcriptase reaction, the mixture was removed from the thermocycler, and 4 μl of 25 mM $MgCl_2$, 8 μl of 10× PCR buffer II, 65.5 μl of H_2O, 0.5 μl of AmpliTaq polymerase at 5 units/μl, 1 μl of 0.4 mM of the forward primer, and 1 μl of 0.4 mM of the reverse primers were added for a total volume of 100 μl. A trace amount of [α-^{32}P]dCTP (1.5 μCi) was added to the reaction. The mixture was then returned to the thermocycler. After 2 min of denaturing at 96°C, the mixture was subjected to 30–35 cycles with the following settings: 96°C for 40 sec, 58°C for 40 sec, and 72°C for 60 sec. Once the cycles were completed, the mixture was held at 4°C. The annealing temperature was modified for the dopamine transporter fragments from 58°C to 62°C. All samples that were not immediately used were stored at −20°C. One-tenth of each PCR product was subjected to electrophoresis on a 2% agarose gel and the dried gel was exposed to x-ray film (Amersham) overnight.

Viability of Grafted Cells. The viability of grafted cells was determined by histologic sections and by their ability to grow *in vitro*. The procedures for growing grafted cells *in vitro* were as described (20).

Evidence of Formation of Neurites by Grafted Cells. This was primarily determined by Bodian staining. The Bodian stain was done to visualize nerve fibers and nerve endings. Briefly, this involves deparaffinizing the slides; soaking at 37°C in 1% Protargol (Roboz Surgical Instruments, Washington, DC) solution for 72 h, rinsing in water, soaking in reducing solution [hydroquinone (10 g/liter)/2% formalin], rinsing in water, toneing in 1% gold chloride, rinsing in water, developing in 2% oxalic acid solution, rinsing in water, treating with 5% sodium thiosulfate, rinsing with water, and counterstaining with aniline blue [aniline blue (0.33 g/liter)/oxalic acid (6.6 g/liter)/phosphomolybdic acid (6.6 g/liter)].

Tumorigenicity and Immunogenicity of Grafted Cells. To determine the possibility of tumor formation in nonlesioned rats receiving transplants of undifferentiated $1RB_3AN_{27}$ cells, gross and histological examinations were performed. Rats that had received two $1RB_3AN_{27}$ transplant tracks were sacrificed at 1, 4, 8, and 13 months after transplantation and perfused with 4% paraformaldehyde. Brains removed, embedded in paraffin, and then cross-sectioned at 10 μm. Paraffin sections of grafted brain were stained with the Harris' hematoxylin/eosin procedure. Briefly, this involves deparaffinizing the sections, staining with Harris' hematoxylin [hematoxylin (5 g/liter)/5% ethanol/ammonium alum (100 g/liter)/mercuric oxide (2.5 g/liter)], washing with H_2O, soaking in ethanol, washing in water, blueing with Scott's blueing agent (Sigma), washing with water and then ethanol, counterstaining with eosin/phloxine solution [eosin Y (1 g/liter)/phloxine (0.01 g/liter)/75% ethanol/0.4% glacial acetic acid], dehydrating with ethanol and xylene, and mounting with resinous medium. The presence of inflammatory cells at the site of transplantation was considered as evidence of rejection.

Unilateral 6-OHDA Lesion. Male Sprague–Dawly rats (250–350 g, Sasco, Omaha, NE) were handled and housed in accordance with University guidelines for animal care. They were anesthetized with equithesin (3.0–4.0 ml/kg, supplemented as necessary) and fixed in a stereotaxic frame. A 28-gauge cannula was lowered to the medial forebrain bundle at two sets of coordinates [from bregma: L 1.5 mm; P −4.3 mm; V 7.5 mm and L 2.0 mm; P −2.3 mm; V 7.8 mm; according to Paxinos and Watson (27)]. Five microliters of 6-OHDA, Br salt (2 mg/ml in saline containing 0.1% ascorbic acid) was infused at 1 μl/min. Afterwards the cannula was left in place for 2 min prior to slow withdrawal.

Transplantation. Differentiated and undifferentiated $1RB_3AN_{27}$ cells and differentiated NBP_2 were suspended in

RPMI medium 1640 at a concentration of 50,000 cells/μl and kept on ice until implantation, which was completed within 5 h. Animals were anesthetized with equithesin (4.0 ml/kg, supplement as necessary) and fixed in a stereotaxic frame that was equipped with a motorized arm (Narishiga, Tokyo). A hole was drilled through the skull (coordinates from bregma: L 2.5 mm; P 0.0 mm) and a 20-gauge stainless-steel guide cannula was lowered 3.5 mm below the dura. The infusion cannula (26-gauge extended 4 mm beyond the end of the guide and was attached to a 25-μl gas-tight syringe (Hamilton) with plastic tubing. Prior to insertion, 4 μl of cell suspension (200,000 cells) were loaded into the infusion cannula and the syringe was placed in a syringe pump. The infusion was made at 1.0 μl/min while the cannula was withdrawn at 1.0 mm/min. After 4 min, the injection was stopped and the cannula was allowed to remain in place for 2 min before withdrawal. Sham-transplanted control animals underwent the same procedure except that they received only RPMI medium 1640.

Neurological Deficits as Measured by Methamphetamine-Induced Turning Rate. Turning rate in response to methamphetamine hydrochloride (5.0 mg/kg, i.p., weight of the salt) was quantified in a flat-bottomed rotometer to assess the efficacy of the lesion produced by 6-OHDA. The rotometer consisted of a Plexiglas cylinder, 20 cm in diameter. The rat was tethered to a counter with a rubber band that was fastened around its chest. Full turns, ipsis- and contralateral to the side of lesion were counted in 10-min blocks for 120 min. Rats that showed a rate of contraversive turning $\geq$3.0 rpm during the period 30–120 min after injection of methamphetamine were used for transplant studies. Previous studies have shown that this rate of turning indicates a neurological deficit where a unilateral DA depletion of more than 95% has occurred (28).

Apomorphine was used to evaluate the level of postsynaptic dopamine receptors in 6-OHDA lesioned rats. This is in contrast to methamphetamine, which invokes a large release of DA. The rate of turning caused by apomorphine (0.05 mg/kg, s.c.) was measured for 30 min after injection in rats prior to transplant and at 1–6 months after transplant. However, apomorphine induced turning rate was not used as criteria for rats receiving transplants.

Statistical Analysis. A randomized analysis (29) appropriate for repeated measures data using a Petrondas–Gabriel-type multiple comparisons procedure (30) was used. The method involves linearly interpolating between the means of the observed rpm measurements for each group and making comparisons of these interpolated curves for various time intervals.

RESULTS

Induction of Morphological Differentiation in $1RB_3AN_{27}$ and NBP_2 Cells. dbcAMP treatment of cells plated on precoated dishes was more effective in inducing morphological differentiation than those plated on regular dishes. The dbcAMP concentration of 2 mM was toxic to cells plated on regular plastic dishes (data not shown). However, it induced morphological differentiation in cells plated on precoated dishes (Fig. 1 *C* and *D*), as evidenced by the formation of neurites, but the level of morphological differentiation varied from one area of the dish to another. One area (Fig. 1*C*) showed a high level of differentiation, whereas another area

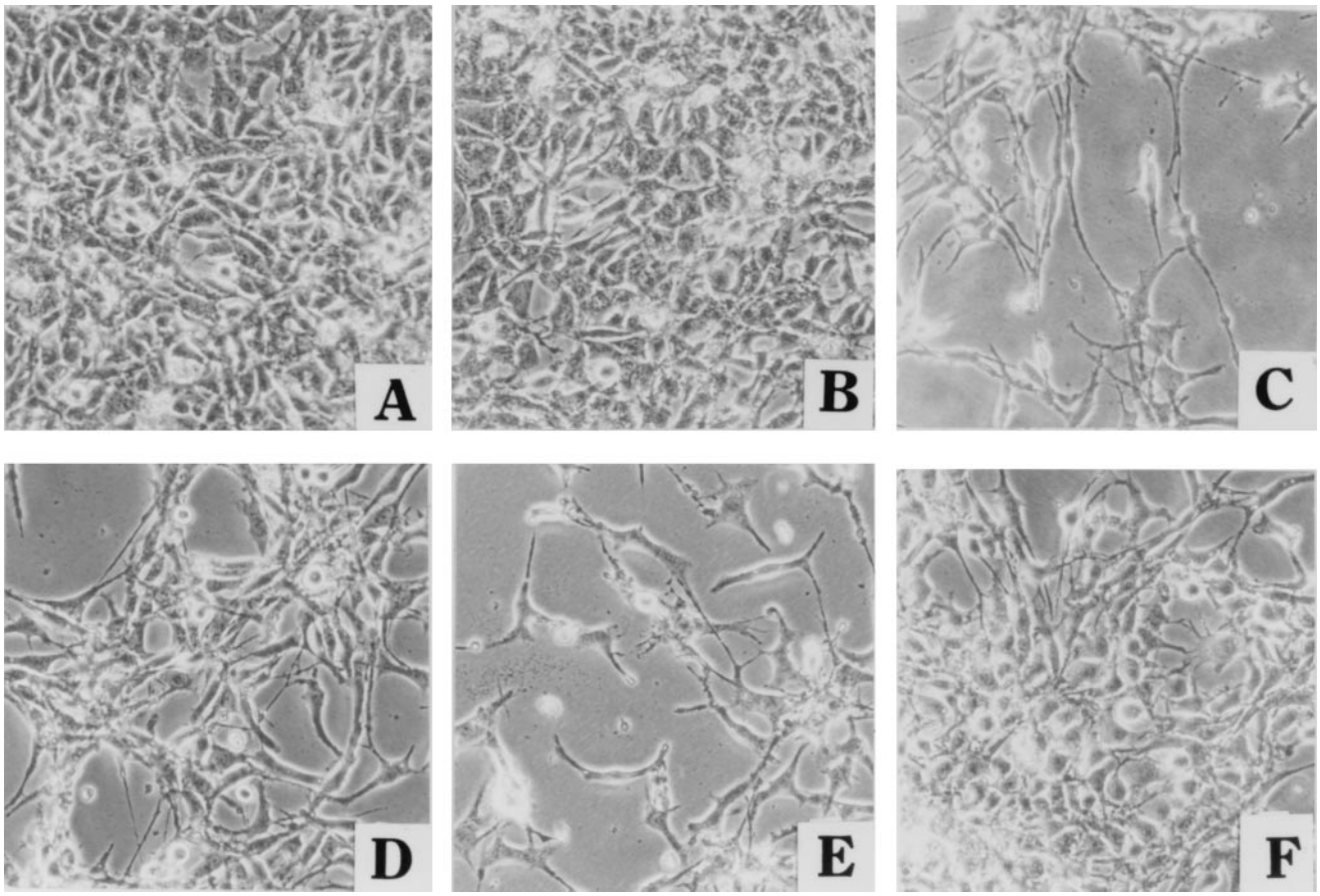

FIG. 1. Photomicrographs of $1RB_3AN_{27}$ cells. Control undifferentiated cells showed cells of various sizes (*A*). Negative control was undifferentiated cells treated with solvent alone (*B*). Cells treated with 2 mm dbcAMP showed areas with high levels of differentiation (*C*) and areas with minimal levels of differentiation (*D*). Cells treated by a combination of 2 mM dbcAMP and DHEA at 60 μg/ml showed areas of high levels of differentiation (*E*) and areas of minimal differentiation (*F*), where most of the cells appear large and round. ($\times$200.)

revealed a lower level of differentiation (Fig. 1*D*). The addition of DHEA enhanced the dbcAMP-induced level of morphological differentiation (Fig. 1 *E* and *F*), whereas DHEA by itself was ineffective (data not shown). The extent of enhancement varied from one area of the dish to another, showing areas with the highest (Fig. 1*E*) and areas with the lowest (Fig. 1*F*) degrees of differentiation. It should be noted that most of the cells which were treated with dbcAMP plus DHEA had become larger (Fig. 1*E*). The solvent treatment did not alter the morphology (Fig. 1*B*) or the growth of cells in comparison to untreated controls (Fig. 1*A*).

The level of differentiation in NBP_2 cells treated with RO20–1724 plus polar carotenoid was similar to that reported earlier (19) (photograph not shown).

Level of TH and DAT mRNAs. TH mRNA was detected in undifferentiated $1RB_3AN_{27}$ cells by using RT–PCR (Fig. 2*A*). TH activity has been demonstrated by biochemical assay in these cells (20). In addition, DAT mRNA was also detectable in undifferentiated $1RB_3AN_{27}$ cells by using RT–PCR (Fig. 2*B*). Differentiation caused an increase in TH mRNA in $1RB_3AN_{27}$ cells and an increase of DAT mRNA in $1RB_3AN_{27}$ cells.

Viability, Immunogenicity, and Innervation of Grafted Cells. The viability of grafted undifferentiated and differentiated $1RB_3AN_{27}$ cells at 8 months after transplant were good as evidence by histological sections (Fig. 3 *A* and *B*) and by their capacity to grow in culture (Fig. 3*C*). There also was no evidence of leukocytic infiltration of the grafted areas. However, there was no evidence of axonal innervation by DA cells because the growth of nerve fibers occurred only along the transplant track, as visualized by Bodian stain (Fig. 3*B*). The viability of differentiated NBP_2 cells was poor, as shown by the fact that there were only a few cells visible in hematoxylin/eosin staining (data not shown).

Tumorigenicity of Grafted Cells. There was no evidence of cell proliferation or tumor formation in 6-OHDA-lesioned animals receiving undifferentiated or differentiated $1RB_3AN_{27}$ cells or differentiated NBP_2 cells at 6 months after transplant (data not shown). There also was no evidence of tumor formation in naive animals at 8 months after transplant (Fig. 3 *A* and *B*) and at 13 months posttransplant (data not shown).

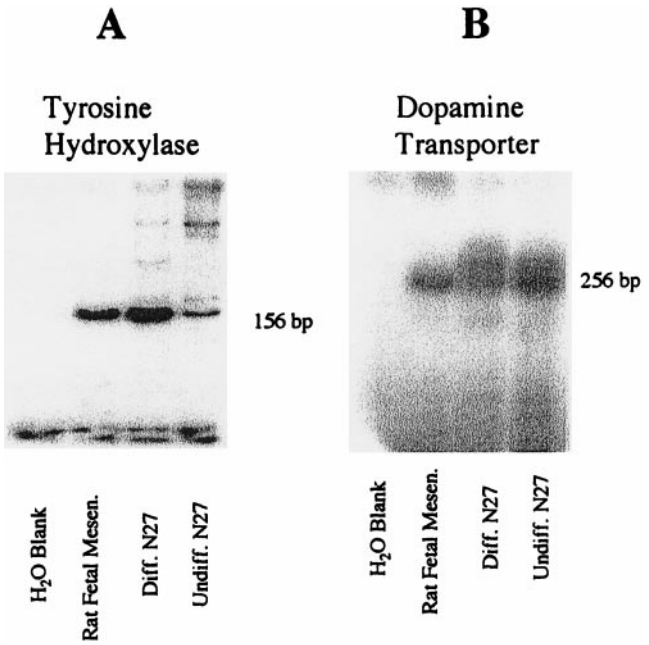

FIG. 2. Level of TH and DAT mRNAs as determined by RT–PCR. Both TH mRNA and DAT mRNA were detectable in undifferentiated $1RB_3AN_{27}$ cells (UN_{27}) and differentiated $1RB_3AN_{27}$ cells (DN_{27}). Rat fetal mesencephalic tissue was a positive control and a water blank was a negative control.

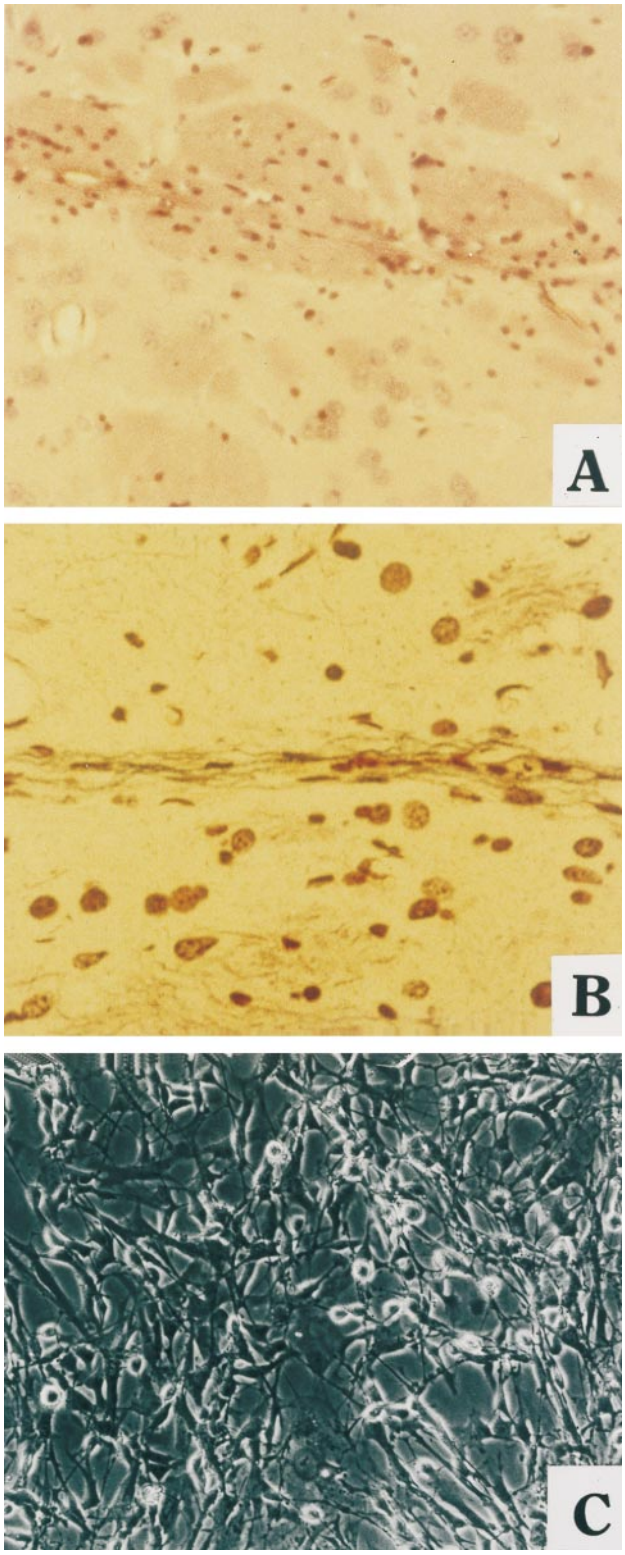

FIG. 3. Photomicrographs of grafted cells in histological sections at 8 months after transplant: hematoxylin/eosin staining (*A*) and Bodian staining (*B*) of the same transplant track. Grafted cells recultured *in vitro* after 13 months of transplantation showed morphology similar to that found before grafting (*C*).

Efficacy of Grafted Cells in Reducing Methamphetamine-Induced Turning Rate in 6-OHDA-Lesioned Rats. Transplan-

tation of differentiated $1RB_3AN_{27}$ cells into striata of 6-OHDA-lesioned rats produced a reduction in methamphetamine-induced turning rate among 100% of transplanted animals at 3 months after grafting, when none of the sham-transplanted animals showed a significant change. Grafting of undifferentiated $1RB_3AN_{27}$ cells and differentiated NBP_2 improved the neurological deficit in 63% and 56% of 6-OHDA-lesioned rats, respectively. Animals receiving differentiated $1RB_3AN_{27}$ cells continued to show improvement in neurological deficits in 100% of transplanted animals, at 4, 5, and 6 months after grafting. However, in sham-transplanted groups, a progressive spontaneous recovery was observed at 6 months after grafting.

At 3 months after transplantation, the extent of reduction of methamphetamine-induced turning rate among the 6-OHDA-lesioned rats that showed improvement in neurological deficits was better in animals receiving differentiated $1RB_3AN_{27}$ (Fig. 4) than those receiving undifferentiated $1RB_3AN_{27}$ cells (Fig. 5). However, the extent of reduction of turning rate was lower in animals receiving grafts of differentiated NBP_2 cells (Fig. 6, $P < 0.05$). The sham-transplanted animals exhibited increased turning during the first 3 months posttransplant. At 6 months after transplantation, the extent of reduction in drug-induced turning rate was most pronounced in 6-OHDA-lesioned animals receiving grafts of differentiated $1RB_3AN_{27}$ cells. The grafting of differentiated $1RB_3AN_{27}$ cells produced greater improvement in neurological deficits than in animals receiving undifferentiated $1RB_3AN_{27}$, differentiated NBP_2 cells, or sham transplanted animals.

The apomorphine-induced turning rate was not altered by grafted differentiated $1RB_3AN_{27}$ cells, undifferentiated $1RB_3AN_{27}$ cells, or differentiated NBP_2 cells in 6-OHDA-lesioned rats (data not shown).

DISCUSSION

This study demonstrates that grafting of LTa gene-induced immortalized DA-producing neurons into striatum improves the neurological deficits in the rat model of PD. The grafting of differentiated $1RB_3AN_{27}$ cells was more effective than the grafting of undifferentiated $1RB_3AN_{27}$ or differentiated NBP_2 cells in improving neurological deficits in 6-OHDA-lesioned rats at 3 months and 6 months after transplantation. This may be due to the fact that differentiated $1RB_3AN_{27}$ cells exhibit increased levels of TH and DAT. Previous studies have shown that the transplantation of a embryonic day 15 mesencephalon (~250,000 total cells) into 6-OHDA-lesioned rats causes improvements in neurological deficits in >80% of the animals at 6 weeks after transplant (31). This value is comparable to that obtained after grafting of 200,000 differentiated $1RB_3AN_{27}$ cells. Nevertheless, the extent of improvement in neurological

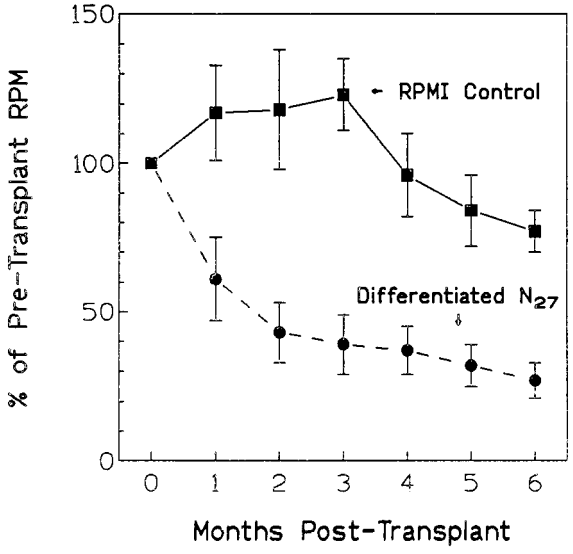

FIG. 4. Reduction of neurological deficit in animals receiving differentiated $1RB_3AN_{27}$ cells. Methamphetamine-induced turning measured in 6-OHDA-lesioned animals as a function of time after grafting of 200,000 differentiated $1RB_3AN_{27}$ cells into the striatum. The rpm value of treated animals at each time period was expressed as percent of pretransplant rpm value. The value at each point represents an average (±SEM) of 8–10 samples. For RPMI control vs. differentiated $1RB_3AN_{27}$ cells, $P < 0.05$.

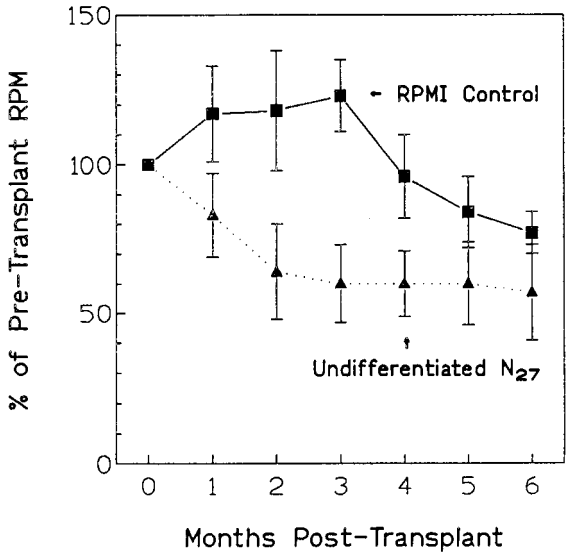

FIG. 5. Reduction of neurological deficit in animals receiving undifferentiated $1RB_3AN_{27}$ cells. Methamphetamine-induced turning measured in 6-OHDA-lesioned animals as a function of time after grafting of 200,000 undifferentiated $1RB_3AN_{27}$ cells into the striatum. The rpm value of treated animals at each time period was expressed as percent of pretransplant rpm value. The value at each point represents an average (±SEM) of 8–10 samples. For RPMI control vs. undifferentiated $1RB_3AN_{27}$ cells, $P < 0.05$.

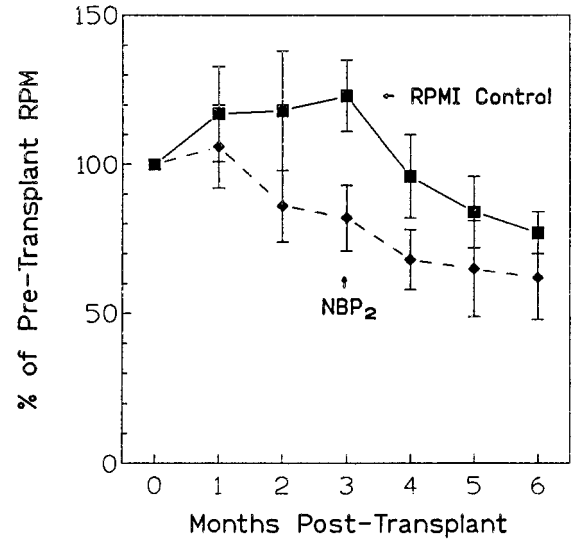

FIG. 6. Reduction of neurological deficit in animals receiving differentiated NBP_2 cells. Methamphetamine-induced turning measured in 6-OHDA-lesioned animals as a function of time after grafting of 200,000 differentiated NBP_2 cells into the striatum. The rpm value of treated animals at each time period was expressed as percent of pretransplant rpm value. The value at each point represents as average (±SEM) of 8–10 samples. For RPMI control vs. NBP_2 cells, $P < 0.05$.

deficits as measured by the reduction in methamphetamine-induced turning rate was much better in animals receiving tissue transplants than those receiving differentiated $1RB_3AN_{27}$ cell transplants. It is not known whether the grafting of increased number of differentiated or undifferentiated $1RB_3AN_{27}$ cells would proportionally increase the extent of improvement in neurological deficits in 6-OHDA-lesioned rats. Most of the previous studies using tissues or established cell lines have limited their observation period up to 3 months (1–7, 17, 18) after transplantation. Our present study shows that the efficacy of grafted $1RB_3AN_{27}$ cells in improving neurological deficits in 6-OHDA-lesioned rats persists at least 6 months after transplantation. It should be noted that at 6 months after transplantation, there was a tendency of spontaneous recovery among sham-transplanted groups.

The grafted $1RB_3AN_{27}$ cells were viable for the entire period of observation, because they can be recultured *in vitro* and their presence can be demonstrated along the transplantation track in hematoxylin/eosin sections. In contrast, only a few differentiated murine NBP_2 cells were detectable in hematoxylin/eosin sections.

Our present study shows that the rate of turning induced by apomorphine (postsynaptic DA stimulation) was not reduced in 6-OHDA-lesioned rats receiving cell transplants. This observation is consistent with the histological observation using Bodian staining that the grafted undifferentiated or differentiated $1RB_3AN_{27}$ cells do not form synapses or neuronal connections. It appears that dopamine released into striatum by diffusion from $1RB_3AN_{27}$ cells is sufficient to improve the neurological deficits in 6-OHDA-lesioned rats. This is analogous to improvement seen in patients with PD after Levodopa therapy, which provides needed DA to the striatum.

One of the major concerns in the transplantation community is that the use of oncogene-induced immortalized cells may not be practical. This is because of the potential danger of malignant transformation of either grafted cells themselves or host cells that may incorporate the oncogene after lysis of grafted cells. Our present observation shows that grafted SV40 LTa gene-induced immortalized DA cells do not form tumors as late as 13 months after transplantation. This observation suggests that such immortalized cells may be grafted into brain without significant danger of tumor formation. This is due to the fact that brain contains LTa gene-inhibiting factors that block the production of LTa and thereby cell proliferation in grafted cells *in vivo* (32).

Another major concern in the transplantation community is the issue of rejection of allogeneic cells or tissue. Allogeneic tissues are prone to rejection because it is difficult to remove all donor-antigen presenting cells primarily responsible for the induction of allo-immunization (12). Although viral oncogene-induced immortalized allogeneic cells are free of donor antigen-presenting cells, they may provoke a host immunological response for rejection due to presence of viral proteins. However, our present observation shows no evidence of any inflammatory response illicited by grafted differentiated or undifferentiated $1RB_3AN_{27}$ cells. These results suggest that SV40 LTa gene-induced immortalized DA neurons may serve as an unlimited source of cells for transplant therapy and should be investigated for their potential use in advanced cases PD. Such cells are neither tumorigenic nor immunogenic as shown in this study.

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