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Immunotoxin-mediated conditional disruption of specific neurons in transgenic mice

(recombinant immunotoxin/dopamine β-hydroxylase/interleukin 2 receptor/cell ablation)

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ABSTRACT **We have developed a transgenic approach, termed immunotoxin-mediated cell targeting (IMCT), to ablate conditionally selective neurons in the brain with the cytotoxic activity of immunotoxins. Transgenic mice were created that express the human interleukin 2 receptor α subunit (IL-2Rα) under the control of the dopamine β-hydroxylase (DBH) gene promoter. The animals were treated intracerebroventricularly with a recombinant immunotoxin, anti-Tac(Fv)-PE40, which selectively kills animal cells bearing human IL-2Rα. The immunotoxin caused a characteristic behavioral abnormality only in the transgenic mice. This was accompanied by a dramatic loss of DBH-containing neurons and a significant decrease in DBH activity and norepinephrine levels in various regions of the brain. IMCT should provide a general technique to create animal models of human neurodegenerative disorders by targeting neurons or other cell types.**

An understanding of the functions of the brain, which depend on neuron–neuron interactions, has progressed with the development of methods that permit the selective elimination of neuronal types with particular identities. When ablation of a subpopulation of neurons leads to physiological and behavioral changes, it is possible to determine the role of a specific type of neuron in brain function. For example, several neurotoxic compounds, such as 6-hydroxydopamine (1) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (2, 3), have been used to deplete selective neurons based on their pharmacological specificities. Also, electrolytic lesioning of small areas containing specified nuclei or axonal bundles has been used to localize functions to a specific system.

Genetic ablation of selective cell types in transgenic animals has provided a powerful tool for studying cell-lineage relationships in mammalian development (4). General methods are based on expression of a toxic gene product during development under the control of a tissue-specific enhancer/promoter (5, 6). An alternative approach is conditional obliteration of target cells with herpes simplex virus 1 thymidine kinase (7–9). This ablation is induced by nucleoside analogs that are converted to toxic compounds by herpes thymidine kinase expressed in transgenic mice. However, this technique cannot be used to eliminate postmitotic cells including neurons, because the toxic compounds induce cell death by inhibiting DNA replication.

Immunotoxins are conjugates of monoclonal antibodies with toxins that kill animal cells bearing appropriate antigens (10). In recombinant immunotoxins, the Fv fragment of the antibody is directly fused to the toxin. These molecules are being evaluated as potential chemotherapeutic agents for the treatment of cancer, autoimmune diseases, and chronic infectious disorders (11). If immunotoxins could kill specific cell types engineered to express target molecules in transgenic mice, they would provide a general technique for conditionally ablating both mitotic and postmitotic cells. Here we describe a cell ablation system, termed immunotoxin-mediated cell targeting (IMCT), to degenerate conditionally specific neuronal types in the central nervous system of transgenic mice.

MATERIALS AND METHODS

Plasmid Construct and Production of Transgenic Mice. The plasmid pDIL was constructed by replacing the cDNA part of pDPN (12) with a cDNA fragment encoding human interleukin 2 receptor α subunit (IL-2Rα) (13). The construct was microinjected into fertilized (C57BL/6J × DBA/2J)F_2 mouse eggs, which were implanted into pseudopregnant female mice. Integration of the transgene was identified by Southern blot analysis of tail DNA.

Intracerebroventricular (i.c.v.) Injection. Anti-Tac(Fv)-PE40 was purified from *Escherichia coli* strain BL21(λDE3) as described (14, 15) and diluted to a final concentration of 40 μg/ml with phosphate-buffered saline (PBS). Male mice (16–20 weeks old) were anesthetized with sodium pentobarbital (Nembutal; 50 mg/kg i.p.) and surgically implanted with a 22-gauge guide cannula into the lateral ventricle 5–7 days before treatment. Administration was carried out through an injection probe placed inside the cannula at a constant velocity of 0.5 $\mu l \cdot min^{-1}$ with a microinfusion pump.

Reverse Transcription (RT)-PCR Analysis. Total RNA was prepared from individual tissues of mice and subjected to RT-PCR analysis (12). For detection of transgene RNA expression, primer A (5′-ATGGATTCATACCTGCTGAT-3′; nucleotides 175–194) and primer B (5′-GTTCCCGGCTTCTTACCAAG-3′; nucleotides 1016–1035) were used (13).

Behavioral Analysis. Animal behavior was monitored by the open-field method (16). Mice treated i.c.v. were individually placed in an open-field apparatus; the floor of the field was divided into 49 equal grids (one grid: 10 × 10 cm). The amount of movement was evaluated by measuring the movement score defined as the number of times the mouse crossed a grid line marked on the floor during a 30-min period. During the same period, the number of falls was also scored. The ratio of the number of falls to the movement score, which was designated as the fall index, was calculated to evaluate the severity of ataxia.

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Abbreviations: DBH, dopamine β-hydroxylase; TH, tyrosine hydroxylase; IL-2Rα, interleukin 2 receptor α subunit; IMCT, immunotoxin-mediated cell targeting; i.c.v., intracerebroventricular; RT, reverse transcription.

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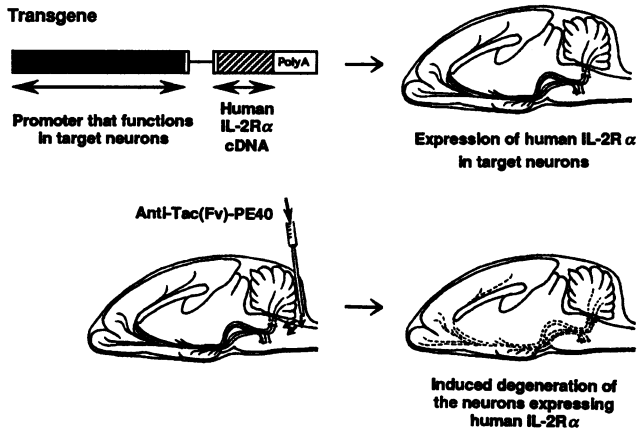

FIG. 1. Strategy of the IMCT procedure for disrupting the target neurons in transgenic mice. The upper part of the figure shows that a transgene is constructed by connecting the human IL-2Rα cDNA downstream of an enhancer/promoter that drives expression in specific neuron types. The human IL-2Rα protein is presented on the cell surfaces of the transgenic target neurons. The lower portion of the figure shows that, subsequently, anti-Tac(Fv)-PE40 is administered i.c.v. to adult transgenic mice and the neurons bearing human IL-2Rα are disrupted by the cell-killing activity of the immunotoxin.

Immunohistochemical Analysis. Frozen sections (40 μm thick) were stained with anti-Tac antibody (1:1000 dilution) or anti-tyrosine hydroxylase (TH) antibody (1:10,000 dilution) by the peroxidase-antiperoxidase method (17). For electron microscopy, ultrathin sections were stained with uranyl acetate and lead citrate.

Biochemical Analysis. Dopamine β-hydroxylase (DBH) activity was determined by HPLC with electrochemical detection, and catecholamine contents were measured with an automatic HPLC system (18).

RESULTS

Strategy for Ablating Specific Neuron Types with Immunotoxins. Our experimental strategy with the IMCT procedure is shown in Fig. 1. We use a recombinant immunotoxin, anti-Tac(Fv)-PE40 (14, 15), in which the variable regions of the anti-Tac antibody, a monoclonal antibody against human IL-2Rα (19), are fused to PE40, a truncated form of *Pseudomonas* exotoxin. This immunotoxin specifically recognizes human IL-2Rα but does not cross-react with murine IL-2R. In the first step, we generate transgenic mice expressing human IL-2Rα under the control of a promoter that functions tissue specifically in the target neurons. In the next step, anti-Tac(Fv)-PE40 is injected into the lateral ventricle in the transgenic brain with a cannula, and the immunotoxin permeates various brain regions through the cerebrospinal fluid. Anti-Tac(Fv)-PE40 is internalized by the neurons expressing the transgene products and these neurons are ablated by inhibition of protein synthesis due to the exotoxin.

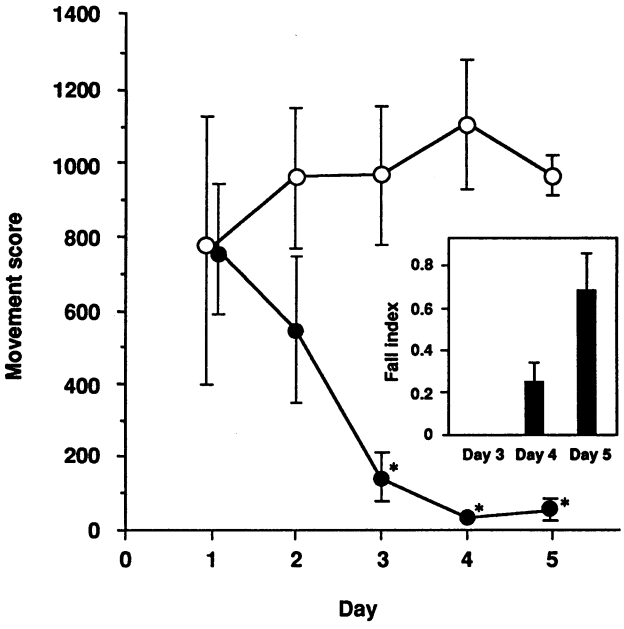

FIG. 3. Analysis of animal behavior after immunotoxin treatment. DIL5-1 transgenic mice and their nontransgenic littermates were treated i.c.v. with the recombinant immunotoxin on day 1, and changes in behavior were monitored every day by the open-field method. Each plot indicates time course of movement scores obtained from both types of mice. ○, Nontransgenic mice; ●, transgenic mice. (*Inset*) Fall index calculated for immunotoxin-injected transgenic mice. Each value represents mean ± SEM of data obtained from four mice. *, Significantly different from nontransgenic mice by Student's *t* test ($P < 0.05$).

Generation of Transgenic Mice and Immunotoxin Treatment. To test the IMCT procedure, we used the 4-kb DNA fragment of the human DBH gene promoter, since our previous studies have shown that this promoter region directs tissue-specific expression of the gene in norepinephrinergic and epinephrinergic neurons as well as in adrenal medullary chromaffin cells (12, 20). Fig. 2*A* shows the transgene construct, which contains the human IL-2Rα cDNA fused downstream of the human DBH gene promoter. Pronuclear microinjection of the construct into fertilized mouse eggs generated

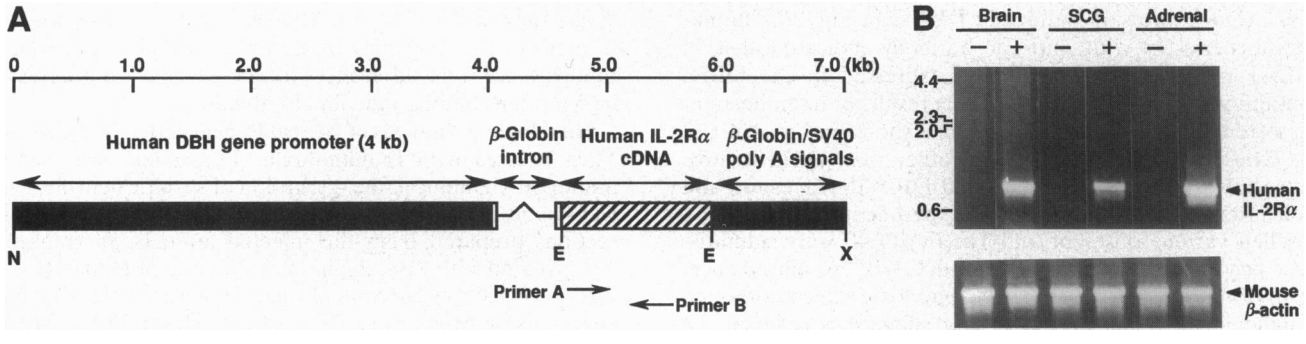

FIG. 2. Creation of transgenic mice expressing human IL-2Rα. (*A*) Structure of transgene construct (pDIL). Construct contains the 4-kb human DBH gene promoter, rabbit β-globin second intron, human IL-2Rα cDNA, rabbit β-globin polyadenylylation signal, and simian virus 40 (SV40) early-gene polyadenylylation signal. Horizontal arrows indicate primers used for RT-PCR analysis. N, *Not* I; E, *Eco*RI; X, *Xho* I. (*B*) Analysis of transgene RNA expression in DIL5-1 transgenic mice. Total RNA was isolated from brain, superior cervical ganglion (SCG), and adrenal medulla of transgenic (lanes +) and nontransgenic (lanes −) mice and subjected to RT-PCR analysis. PCR amplification was carried out with either the transgene primer set or the mouse β-actin primer set.

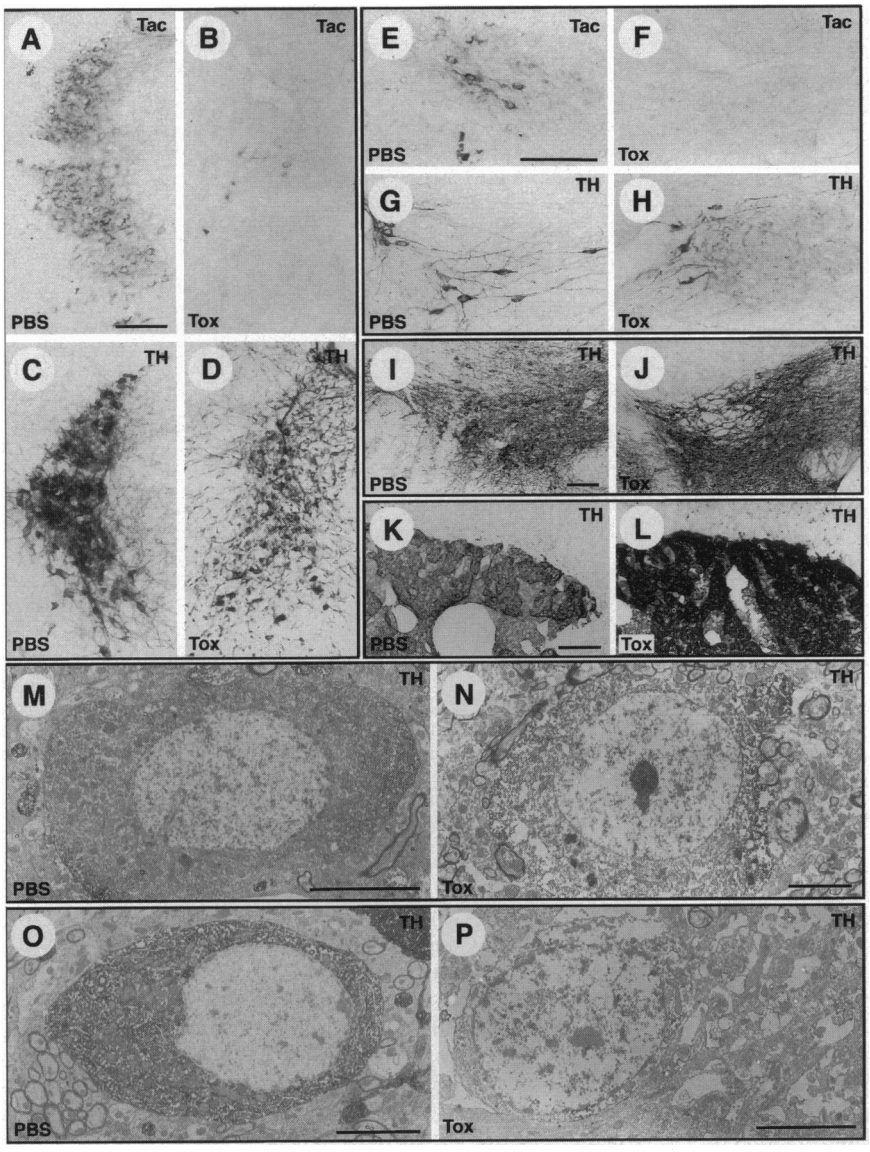

FIG. 4. Immunohistochemical staining of tissue sections from DIL transgenic mice. Light microscopic image of the locus coeruleus or A6 neurons (*A–D*), nucleus tractus solitarius or A2 neurons (*E–H*), ventral tegmental area or A10 neurons (*I* and *J*), and adrenal medullary cells (*K* and *L*). Immunoelectron microscopic image of the A6 (*M* and *N*) and A2 (*O* and *P*) neurons. DIL transgenic mice were treated with PBS or anti-Tac(Fv)-PE40. At day 5 after i.c.v. injection, tissues were removed from the PBS-injected mice (*A, C, E, G, I, K, M,* and *O*) and the immunotoxin-injected mice (*B, D, F, H, J, L, N,* and *P*). Frozen tissue sections were stained with anti-Tac antibody (*A, B, E,* and *F*) or anti-TH antibody (*C, D,* and *G–P*). (*A–L*, bars = 100 μm; *M–P*, bars = 5 μm.)

four founder mice carrying 50–100 copies of transgene per mouse genome. To detect expression of transgene RNA, we carried out RT-PCR analysis of total RNA prepared from brain, superior cervical ganglion, and adrenal gland of transgenic offspring. Human IL-2Rα mRNA was detected in these tissues in four transgenic lines. One transgenic line, designated DIL5-1, showed the highest expression level of transgene RNA. (See expression pattern in DIL5-1 in Fig. 2*B*.) Immunohistochemistry with anti-Tac antibody indicated that in DIL5-1 mice human IL-2Rα was expressed in the DBH-containing neurons in the brain. (The results of immunostaining were basically the same as the data shown in Fig. 4 *A* and *E*). Expression in ectopic regions other than DBH-positive neurons was not apparent. We used DIL5-1 transgenics and their littermates for the following experiments.

When various doses of anti-Tac(Fv)-PE40 were administered i.c.v. as a single injection to adult C57BL/6J inbred mice, doses of <0.3 μg per mouse showed no toxic effects, although a higher dose of 0.5 μg per mouse sometimes reduced the amount of movement. We therefore injected 0.2 μg of anti-Tac(Fv)-PE40 per mouse as a nontoxic dose. All nontransgenic mice treated with the immunotoxin remained healthy for at least 2–3 months, whereas the immunotoxin-injected transgenics gradually developed characteristic behavioral abnormalities. Changes in behavior were monitored with the open-field method as described in *Materials and Methods* (Fig. 3). The first abnormality was a marked decrease in locomotion at day 3 after injection and was followed by ataxic behavior between days 3 and 4. The ataxic behavior was characterized by gait disturbance, frequent falls, and stiffness in the hindlimbs. Subsequently, these animals developed more progressive symptoms along with a decrease in body weight after day 5, and they finally died. The abnormal behavior was observed in almost all transgenics treated with the immunotoxin, although there were differences in the severity and progression in symptoms among individual animals.

Histological Analysis of Neural Degeneration in Transgenic Mice Treated with Immunotoxin. To examine whether the immunotoxin induced the disruption of specific neurons in the transgenic mice, we analyzed immunohistochemically tissue sections prepared from the injected animals. In transgenic mice treated with PBS, the immunostaining of human IL-2Rα was localized in norepinephrinergic neurons in the locus coeruleus or A6 neurons (Fig. 4*A*). As shown in Fig. 4*C*, the locations of these neurons were confirmed by staining with the antibody raised to TH, which is the initial and rate-limiting enzyme of catecholamine synthesis. Immunotoxin administration to transgenic mice caused a dramatic decrease in both human IL-2Rα and TH immunoreactivities in these neurons (Fig. 4 *B* and *D*) with a decrease in the number of cell bodies

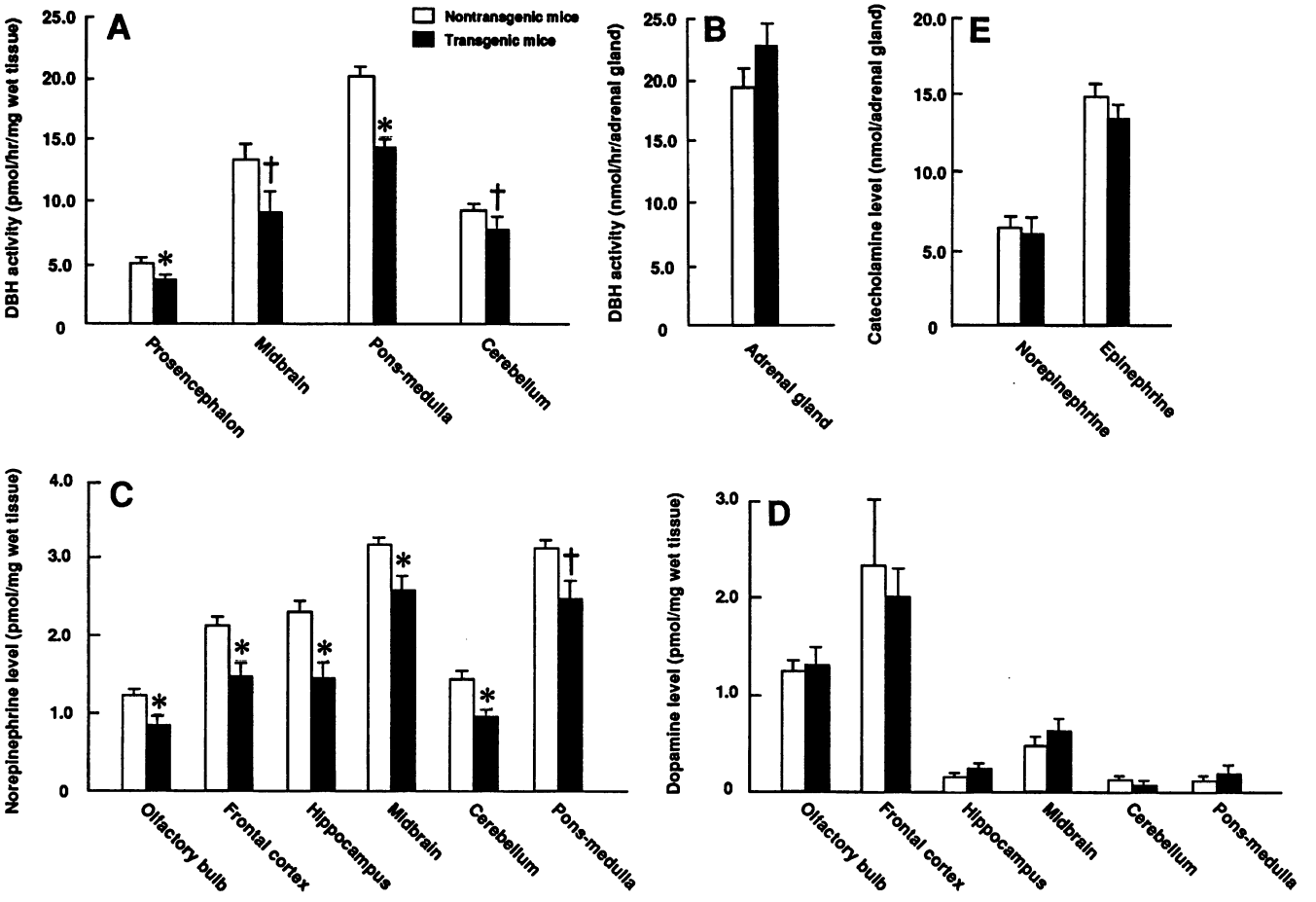

FIG. 5. DBH activity and catecholamine level in mice treated with immunotoxin. Transgenic and nontransgenic littermates were treated with anti-Tac(Fv)-PE40. At day 5 after treatment, tissues were dissected from injected mice. (*A*) DBH activity in various brain regions. (*B*) DBH activity in adrenal gland. (*C* and *D*) Norepinephrine and dopamine levels in various brain regions. (*E*) Catecholamine level in adrenal gland. Each value represents mean ± SEM of data obtained from 9–11 treated mice. Significant differences from nontransgenics treated with immunotoxin according to Student's *t* test: *, $P < 0.01$, †, $P < 0.05$.

and axon fibers. Similar results were obtained for other norepinephrinergic neurons in the nucleus tractus solitarius or A2 neurons (Fig. 4 *E–H*). On the other hand, in dopaminergic neurons of the ventral tegmental area (A10 neurons) and in the adrenal medulla chromaffin cells of the transgenics, the staining pattern with the anti-TH antibody in the immunotoxin-injected animals (Fig. 4 *J* and *L*) was similar to that in the PBS-injected animals (Fig. 4 *I* and *K*). In nontransgenic mice, the immunotoxin treatment did not cause any visible change in TH immunoreactivity in all catecholaminergic neurons and adrenal medullary cells (data not shown).

Immunoelectron microscopy was used to examine the intracellular structure of A6 and A2 neurons affected by anti-Tac(Fv)-PE40. The PBS-injected transgenic mice showed a normal staining pattern with strong TH immunoreactivity present in the cytoplasm (Fig. 4 *M* and *O*). In contrast, dramatic morphological changes in intracellular structure were observed in the immunotoxin-treated transgenic mice (Fig. 4 *N* and *P*) with disorganization of nuclear chromatin structure, vacuolation of the cytoplasm, and diminished TH immunoreactivity in the cytoplasm.

Effects on Biochemical Parameters. DBH activity and catecholamine level in representative tissues were compared between DIL transgenic and nontransgenic littermates treated with anti-Tac(Fv)-PE40 (Fig. 5). In various brain regions of the immunotoxin-treated transgenic mice, DBH activity was significantly lower than the values for the corresponding nontransgenic tissues; decreases were 82% in prosencephalon, 62% in midbrain, 74% in pons medulla, and 86% in cerebellum (Fig. 5*A*), and norepinephrine level was also reduced to 70% in olfactory bulb, 71% in frontal cortex, 63% in hippocampus, 80% in midbrain, 62% in cerebellum, and 82% in pons medulla as compared with control values (Fig. 5*C*). In contrast, the dopamine level in the brain regions of the injected transgenics was similar to that in the corresponding nontransgenic tissues (Fig. 5*D*). Also, there was no statistically significant difference in DBH activity, norepinephrine level, and epinephrine level in adrenal gland between transgenic and nontransgenic mice treated with immunotoxin (Fig. 5 *B* and *E*).

DISCUSSION

A transgenic mouse technology for conditional cell ablation was designed on the basis of the species-specific action of anti-Tac(Fv)-PE40. To express the target molecule for anti-Tac(Fv)-PE40 in specific cell types, we generated transgenic mice carrying a chimeric gene that contains a human IL-2Rα cDNA downstream of the human DBH gene promoter. A single i.c.v. injection of 0.2 μg of anti-Tac(Fv)-PE40 was nontoxic to nontransgenic control animals, and no existence of toxicity was observed upon histological and biochemical analyses of these animals. When the transgenic mice were treated with 0.2 μg of anti-Tac(Fv)-PE40, they developed abnormal behavior beginning on day 3 after treatment accompanied by a dramatic loss of DBH-containing neurons and a significant decrease in DBH activity and norepinephrine levels in the brain. These results demonstrate that it is possible to destroy

specific cell types in transgenic mice engineered to express target molecules with a recombinant immunotoxin.

Norepinephrinergic fibers, which originate from several nuclei present in pons medulla, project to a wide range of brain regions, including olfactory bulb, frontal cortex, hippocampus, and cerebellum (21). In all the brain regions we examined, both DBH activity and norepinephrine levels were greatly decreased in the immunotoxin-injected transgenic animals, indicating that the immunotoxin influences catecholamine metabolism in both the cell body and the nerve terminal region of norepinephrinergic neurons. These observations are consistent with the results obtained from histological studies that show degeneration and loss of norepinephrinergic cell bodies and their fibers. In contrast to the effects of anti-Tac(Fv)-PE40 on the brain, the immunotoxin did not show any cytotoxic activity against adrenal medullary chromaffin cells of transgenic animals, possibly because the immunotoxin molecules injected i.c.v. cannot efficiently cross the blood–brain barrier.

Behavioral functions of norepinephrinergic neurons in the central nervous system have been characterized by using electrolytic lesions, chemical depletion, and single unit recording techniques (22, 23). We observed a characteristic behavioral abnormality in the transgenic mice after immunotoxin treatment. Because the immunotoxin probably contacts cells throughout the brain, it may produce more severe and extensive effects on animals compared to other studies of norepinephrinergic functions. Ataxic behavior has not been reported to be caused by norepinephrinergic deficits, although it is known that norepinephrinergic fibers form synapses on Purkinje cells in the cerebellar cortex and regulate the spontaneous firing of these neurons (24, 25). To explain the behavioral abnormalities observed, we must also consider the possibility of transgene expression in ectopic regions, where the DBH gene is not normally expressed. Ectopic expression of transgene products occasionally occurs in transgenic mice and may be due to position effects around the transgene integration site (26, 27) or to some other effect of the promoter and the reporter gene (28). Our immunohistochemical analysis of DIL5-1 transgenic mice with the anti-Tac antibody did not detect expression of human IL-2Rα in ectopic sites, but we cannot exclude the possibility that there is a low level expression of transgene products in cells that are not DBH positive. Action of the immunotoxin on cells ectopically expressing the target molecules in addition to the DBH-containing neurons may lead to complex changes in animal behavior. The behavioral abnormalities generated in the transgenic mice after immunotoxin treatment will be further characterized. One approach will be to study the effects of anti-Tac(Fv)-PE40 on cells other than catecholaminergic neurons to get a better understanding of norepinephrinergic functions in the brain.

In this study, the IMCT procedure was applied to eliminate the selective neurons from the brain neural network. The technique provides an approach to elucidate the physiological and behavioral functions of specific neuronal types in the mammalian central nervous system and to create experimental models of human brain disorders, which are known to be caused by degeneration of selective neurons, such as Alzheimer and Huntington diseases. Since the cytotoxicity of immunotoxins is based on their inhibitory effect on protein synthesis, this procedure should be applicable to other cell types in addition to neurons.

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