An Adenovirus Vector for Gene Transfer into Neurons and Glia in the Brain

G. Le Gal La Salle, J. J. Robert, S. Berrard, V. Ridoux, L. D. Stratford-Perricaudet, M. Perricaudet, J. Mallet*

The efficient introduction of genetic material into quiescent nerve cells is important in the study of brain function and for gene therapy of neurological disorders. A replication-deficient adenoviral vector that contained a reporter gene encoding β-galactosidase infected rat nerve cells in vitro and in vivo. β-Galactosidase was expressed in almost all sympathetic neurons and astrocytes in culture. After stereotactic inoculations into the rat hippocampus and the substantia nigra, β-galactosidase activity was detected for 2 months. Infected cells were identified as microglial cells, astrocytes, or neurons with anatomical, morphological, and immunohistochemical criteria. No obvious cytopathic effect was observed.

The ability to deliver foreign genes and promoter elements directly to terminally differentiated cells of the nervous system, which no longer proliferate, would be desirable for the study of the function and regulation of cloned genes as well as for gene therapy. Although a possibility is offered by defective herpes simplex virus vectors (*1*), their usefulness has been limited by their poor efficiency of infection and their pathogenicity. Here, we show that adenovirus, whose natural target is not the nervous system but the respiratory epithelium (*2*), has the ability to infect nerve cells. The gene transfer and expression of adenovirus are highly efficient both in vitro and in the intact rat brain.

In addition to nonreplicative infection, adenovirus has several assets (*3*). Its genome can accommodate foreign genes of up to 7.5 kb. It has a large host range and low pathogenicity in humans, and high titers of the virus can be obtained (*4*). We used a replication-defective adenovirus, Ad.RSV-βgal, which expressed a nuclearly targeted β-galactosidase (β-gal) cDNA under control of the Rous sarcoma virus long terminal repeat (RSV LTR) promoter (*5*). We tested the ability of this vector to infect primary cultures of sympathetic neurons of superior cervical ganglia (SCG). These cells, cultured in the presence of an antimitotic agent, provided a pure and homogeneous preparation of neurons (*6*). After inoculation of the virus, virtually all cells were positive for β-gal activity (*7*), with no apparent toxic effects or morphological changes (Fig. 1, A and B). Labeled cells were not detected when the staining reaction was performed on a parallel, noninoculated culture (Fig. 1C). We also tested the ability of adenovirus to infect primary cultures of rat hippocampal tissue that were enriched in astrocytes (*8*). Inoculation resulted in a blue nuclear staining in about two-thirds of the cells (Fig. 1D). The identification of stained cells as astrocytes was confirmed by additional staining with an antibody against glial fibrillary acidic protein (GFAP) (Fig. 1E).

We next evaluated the ability of adenovirus to infect cells of the brain in vivo in two regions, the hippocampus and the substantia nigra (*9*). All injected animals expressed β-gal activity and β-gal protein, which were detected as early as 24 hours after inoculation and also in animals analyzed after 2 months. The diffusion of the virus was greater in the hippocampus than in the substantia nigra. Infected cells were found throughout the entire dorsal region of the hippocampus (Fig. 2), whereas in the substantia nigra the overall pattern of infection was restricted mainly to a medial-lateral orientation (Fig. 3D). This difference may reflect the propensity of the virus to spread through tissues that adhere loosely, such as the hippocampal fissure.

The extent of the infected area was correlated to the volume of viral solution administered. For instance, in rats killed 3 to 7 days after hippocampal inoculation, the infected area was 1 to 4 mm^3 for 3 to 5 μl of virus injected [10^{10} plaque-forming units (PFU) per milliliter]. Only minor differences in the distribution of the labeling were noted within the first week after inoculation (Fig. 2, A and B). At a longer time, however, the extent of the infected area was more restricted and the labeling was confined to the granule cell layer (Fig. 2C).

No cytotoxic effects in the infected animals were apparent. All recovered from the inoculation procedure without behavioral abnormalities. Examination of the virus-infected brains revealed no enlargement of the lateral ventricle or disruption of the normal anatomy of the structures. The only noticeable alteration was local tissue necrosis and reactive gliosis that were restricted to the injected sites. This phenomenon was largely a result of injection trauma because a similar alteration was observed in animals that had been injected with saline. Finally, analysis of hippocampal cells with Nissl staining showed no cell loss or evidence of cytolysis within the pyramidal or granule cell layers.

We then characterized the infected cell types. At early times (1 to 7 days), many of the β-gal–stained cells exhibited a morphology characteristic of microglial cells in both regions. These small cells had fine, highly branched processes extending radially from the cell body (Fig. 3, A to C). Their identification as microglial cells was confirmed by additional labeling with the antibody OX42, which is directed against type 3 complement receptors (*10*), and with B4-isolectin (*11*). Some of the infected cells were astrocytes, as demonstrated by double staining with the X-gal substrate and an antibody directed against GFAP.

We next determined whether neurons also were infected in both cerebral regions. In the substantia nigra, double-labeling experiments demonstrated coexpression of β-gal and immunoreactivity for tyrosine hydroxylase (TH), a classical marker of catecholaminergic neurons (Fig. 3D). About 50% of the β-gal–positive cells within the infected dopaminergic cell area were marked

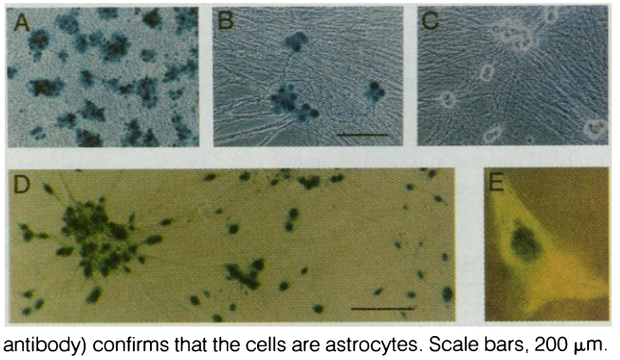

Fig. 1. Expression of β-gal in primary cultured cells after inoculation by adenovirus Ad.RSVβgal. (**A** and **B**) Virtually all the SCG neurons (*13*) expressed β-gal. (**C**) In the absence of the virus, no labeling was observed. (**D**) In enriched astroglial cultures (*14*), about two-thirds of the cells were labeled. (**E**) Additional staining with GFAP (Dakopatts, Glostrup, Denmark, 1:500 dilution, fluorescein-conjugated secondary antibody) confirms that the cells are astrocytes. Scale bars, 200 μm.

G. Le Gal La Salle and V. Ridoux, Institut Alfred Fessard, Unité Propre de Recherche 2212, Centre National de la Recherche Scientifique (CNRS), 91198 Gif sur Yvette, France.
J. J. Robert, S. Berrard, J. Mallet, Laboratoire de génétique moléculaire de la neurotransmission et des processus dégénératifs, C 9923, CNRS, 91198 Gif sur Yvette, France.
L. D. Stratford-Perricaudet and M. Perricaudet, Institut Gustave Roussy, CNRS Unité Associée 1301, 94805 Villejuif Cedex, France.
*To whom correspondence should be addressed.

with a TH antibody, which thereby demonstrates that dopaminergic neurons were infected (Fig. 3, E to G). In the hippocampus, which is composed of segregated and laminated cellular subgroups, numerous β-gal–stained cells were unambiguously identified as neurons on the basis of morphological and anatomical characteristics (Fig. 3, H to J). Recognition of the cellular type was further facilitated because a few cells revealed a Golgi-like profile as a result of the diffusion of β-gal enzyme. These positively stained cells could be identified as pyramidal neurons, granule cells, and hilar interneurons in the pyramidal cell layer CA1, the granule cell layer, and the hilus of the dentate gyrus, respectively (Fig. 3, H to J).

In rats killed 1 and 2 months after inoculation, the distribution of β-gal–positive cells was more restricted than what had been observed at earlier times. Although microglial cells represented a large number of β-gal–expressing cells up to 1 week after injection into the hippocampus, their number decreased at longer post-infection times. Most of the labeled cells at 1 month were neurons of the stratum granulosum (Fig. 4). As determined in sections counterstained with cresyl violet, β-gal–positive cells were restricted to the granular layer, and no positive cells were seen in the innermost part of the layer that includes most of the basket cells and a few glial cells. The same pattern was also obtained at 2 months. This restriction in the pattern may reflect a change in the RSV LTR promoter activity; the activity of the RSV LTR promoter may be more

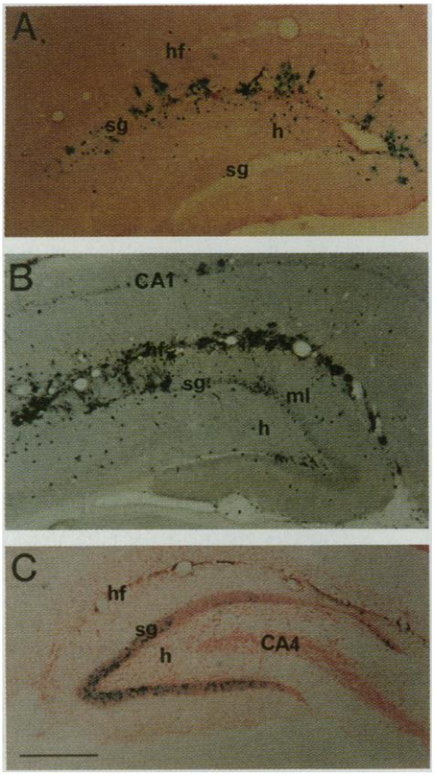

Fig. 2. General patterns of β-gal expression after unilateral intrahippocampal inoculation of the virus Ad.RSVβgal. (**A**) Staining with X-gal and fushin (Gurr, England) in a 40-μm-thick frontal section of the brain of a rat killed 24 hours after injection. (**B**) Immunohistochemical detection of β-gal 1 week after injection. The primary antibody was an affinity-purified rabbit immunoglobulin G fraction to β-gal (Cappel, Organon, West Chester, Pennsylvania, 1:800 dilution) that was then bound with a streptavidin-biotinylated peroxidase complex (Amersham) with diaminobenzidine as a chromogen, reinforced with nickel. (**C**) Distribution of β-gal–positive blue cells in the dentate gyrus of the hippocampus 1 month after injection. Counterstaining is shown in neutral red. Scale bar, 300 μm; h, hilus; hf, hippocampal fissure; ml, molecular layer; sg, stratum granulosum.

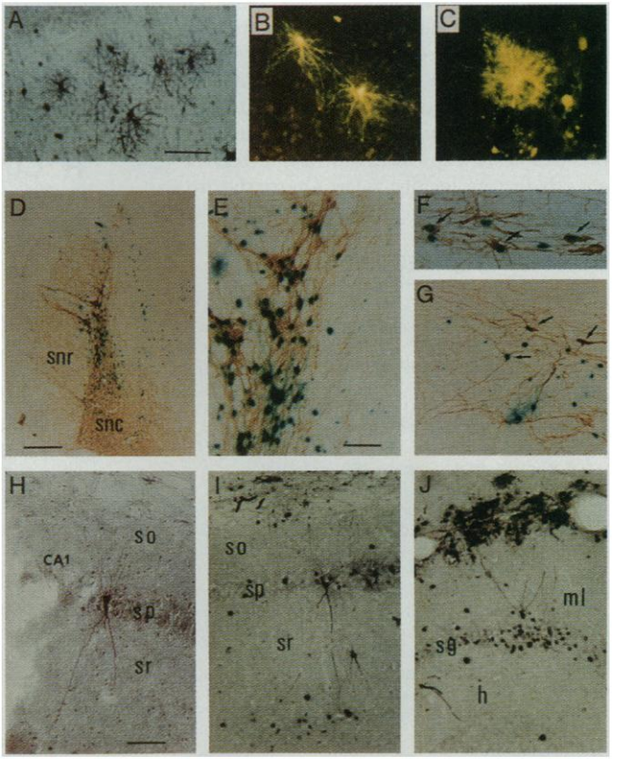

Fig. 3. Characterization of glial and neuronal cell types infected by direct in vivo inoculation of the adenovirus Ad.RSVβgal. (**A** to **C**) Immunohistochemical detection of β-gal expression in microglial cells 5 days after the injection into the hippocampus. Immunological reaction was processed with peroxidase reinforced with nickel (A) and with fluorescein-conjugated secondary antibody (B and C). (**D** to **G**) Sections across the substantia nigra. Three days after intranigral inoculation, a dense blue β-gal staining was detectable in a great number of nigral cells (D), most of which were also shown to be double-labeled with TH monoclonal antibodies (E to G) (Boehringer Mannheim, 1:200 dilution). Panel (E) is a higher magnification of the area with the highest density of β-gal–positive cells in (D). Arrows in (F) and (G) indicate cells double-stained for β-gal and TH. (**H** to **J**) Sections across the hippocampus. Pyramidal cells in CA1 (H and I) and granule cells in the dentate gyrus (J) are labeled. β-gal activity was revealed by immunohistochemistry as in (A). The cells in (D) to (G) were processed with histochemistry; in addition, the cells in (D) to (J) were labeled with antibodies. Scale bars: 30 μm in (A), 300 μm in (D), and 100 μm in (E) and (H). Abbreviations are as in Fig. 2 except for snc, substantia nigra pars compacta; snr, substantia nigra pars reticulata; sp, stratum pyramidale; so, stratum oriens; and sr, stratum radiatum.

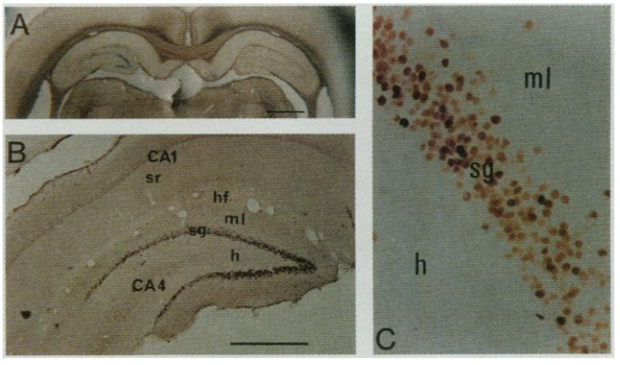

Fig. 4. Distribution of β-gal–positive cells in the dentate gyrus 1 month after Ad.RSVβgal inoculation. (**A**) Photomicrograph stained for β-gal expression with X-gal histochemistry. The cells that are stained blue were observed in the dentate gyrus of the injected left hippocampus. Scale bar, 1 mm. (**B**) Dentate localization of infected cells was confirmed by immunohistochemical β-gal detection (staining with peroxidase plus nickel). Scale bar, 300 μm. (**C**) High magnification view showing the large number of densely packed, β-gal–labeled cell nuclei in the granule cell layer of the dentate gyrus. Abbreviations are as in Fig. 3.

stable in neurons than in glial cells. Another possibility is that the virus may be transferred from one cell type to another, as has been described for rabies and herpes viruses (*12*).

The use of adenovirus vectors provides a method to study the function of cloned genes, which is complementary to that of transgenic animals. For instance, infection of the hippocampus would be useful for the study of integrated phenomena such as long-term potentiation. The possibility of selecting the time at which a particular gene is to be expressed is important when the expression of a transgene in early development is deleterious to the animal. In the context of degenerative diseases, it may also be possible to express neurotransmitters or growth factors locally as an alternative to the grafting of fetal cells.

REFERENCES AND NOTES

1. A. I. Geller and X. O. Breakefield, *Science* **241**, 1667 (1988); A. I. Geller and A. Freese, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 1149 (1990); E. A. Chiocca *et al.*, *New Biol.* **2**, 739 (1990); A. T. Dobson, T. P. Margolis, F. Sedarati, J. G. Stevens, L. T. Feldman, *Neuron* **5**, 353 (1990); D. J. Fink *et al.*, *Hum. Gene Ther.* **3**, 11 (1992).
2. M. A. Rosenfeld *et al.*, *Cell* **68**, 143 (1992); M. A. Rosenfeld *et al.*, *Science* **252**, 431 (1991).
3. L. D. Stratford-Perricaudet and M. Perricaudet, in *Human Gene Transfer*, O. Cohen-Haguenauer, M. Boiron, J. Libbey, Eds. (Eurotext Ltd., London, 1991), pp. 51–61.
4. L. D. Stratford-Perricaudet, M. Levrero, J. F. Chasse, M. Perricaudet, P. Briand, *Hum. Gene Ther.* **1**, 241 (1990); B. Quantin, L. D. Perricaudet, S. Tajbakhsh, J. L. Mandel, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 2581 (1992).
5. L. D. Stratford-Perricaudet, I. Makeh, M. Perricaudet, P. Briand, *J. Clin. Invest.* **90**, 626 (1992).
6. E. Hawrot and P. H. Patterson, *Methods Enzymol.* **58**, 574 (1979).
7. Histochemical staining was performed with the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) as described [J. R. Sanes, J. L. Rubenstein, J. F. Nicolas, *EMBO J.* **5**, 3133 (1986)].
8. K. D. McCarthy and J. De Vellis, *J. Cell Biol.* **85**, 890 (1980).
9. Seventeen male Wistar rats (10 weeks old) were stereotactically injected under deep anesthesia with 1 to 5 μl of media that contained highly purified virus (10^{10} PFU/ml) into either the hippocampus or the substantia nigra. Animals were killed 1, 2, 3, 5, 7, 30, and 60 days after inoculation. We detected β-gal activity in positive cells histochemically by using both the X-gal substrate and an antibody directed against the protein. The latter method is more sensitive and in some instances revealed fine cytoplasmic processes.
10. A. P. Robinson, T. M. White, D. W. Mason, *Immunology* **57**, 239 (1986).
11. C. E. Hayes and I. J. Goldstein, *J. Biol. Chem.* **249**, 1904 (1974).
12. F. Lafay *et al.*, *Virology* **183**, 320 (1991); K. Kristensson, B. Ghetti, H. M. Wisniewski, *Brain Res.* **69**, 189 (1974); X. Martin and M. Dolivo, *ibid.* **273**, 253 (1983).
13. The SCG were removed from 2-day-old Wistar rats, dissociated, plated on 16-mm collagen-coated dishes, and cultured as described (*7*). Cytosine arabinofuranoside (10 μM) was added during the first week of culture to prevent proliferation of ganglionic non-neuronal cells. After 6 days in culture, the cells were inoculated with 10^6 PFU of Ad.RSVβgal in culture medium or, as a control, exposed only to culture medium. Twenty-four hours later, the virus was removed, and the cells were maintained for 2 days in culture medium. After washing and paraformaldehyde fixation, β-gal–expressing cells were characterized with X-gal histochemistry.
14. Cells were plated in 35-mm-diameter plastic dishes and grown in supplemented Dulbecco's modified Eagle's medium for 5 days. The cells in each dish were then inoculated with 2 μl of the adenoviral solution (titer, 10^8 PFU/ml) for 24 hours. Histochemical staining was processed as in (*13*).

15. The first two authors contributed equally to this paper. We thank N. Faucon Biguet for helpful discussions, A. Hicks and C. Menini for critically reading this manuscript, and M. Synguelakis and S. Mirman for technical assistance. Supported by grants from CNRS, the Association pour la Recherche contre le Cancer, the Association Française contre la Myopathie, the Institut de Recherche sur la Moelle Epinière, and the Bioavenir Program (Rhône-Poulenc Rorer, Ministère de la Recherche et de l'Espace, and Ministère de l'Industrie et du Commerce Extérieur). J.J.R. received a fellowship from the Institut de Formation Supérieure Biomédicale.

4 September 1992; accepted 18 December 1992

CD40 Ligand Gene Defects Responsible for X-Linked Hyper-IgM Syndrome

R. Cutler Allen, Richard J. Armitage, Mary Ellen Conley, Howard Rosenblatt, Nancy A. Jenkins, Neal G. Copeland, Mary A. Bedell, Susanne Edelhoff, Christine M. Disteche, Denise K. Simoneaux, William C. Fanslow, John Belmont, Melanie K. Spriggs*

The ligand for CD40 (CD40L) is a membrane glycoprotein on activated T cells that induces B cell proliferation and immunoglobulin secretion. Abnormalities in the CD40L gene were associated with an X-linked immunodeficiency in humans [hyper-IgM (immunoglobulin M) syndrome]. This disease is characterized by elevated concentrations of serum IgM and decreased amounts of all other isotypes. CD40L complementary DNAs from three of four patients with this syndrome contained distinct point mutations. Recombinant expression of two of the mutant CD40L complementary DNAs resulted in proteins incapable of binding to CD40 and unable to induce proliferation or IgE secretion from normal B cells. Activated T cells from the four affected patients failed to express wild-type CD40L, although their B cells responded normally to wild-type CD40L. Thus, these CD40L defects lead to a T cell abnormality that results in the failure of patient B cells to undergo immunoglobulin class switching.

Human hyper-IgM immunodeficiency is a rare disorder characterized by normal or elevated serum concentrations of polyclonal IgM and markedly decreased concentrations of IgA, IgE, and IgG (*1*, *2*). Reports of X-linked, autosomal recessive, autosomal dominant, and acquired forms of the disorder indicate genetic heterogeneity and that several different pathologic mechanisms may be responsible (*2*, *3*). In the X-linked form of hyper-IgM syndrome, affected males usually experience the onset of recurrent infections in the first year of life. Affected males have normal numbers of circulating B and T lymphocytes, although lymph node hyperplasia with an absence of germinal centers is common (*1*, *2*). This condition is lethal in the absence of medical intervention; however, patients typically respond well to a maintenance therapy consisting of intravenous treatment with γ globulin.

The cellular abnormalities that underlie the various forms of hyper-IgM syndrome are unclear. Studies of patterns of X chromosome inactivation in obligate carrier females indicate a randomized pattern of X chromosome usage in either B or T lineage cells, which suggests that the defect does not alter maturation of these cells by cell autonomous mechanisms (*4*). Some studies have suggested that the affected phenotype is likely a result of B cell dysfunction insofar as patient B cells treated with polyclonal B cell activators, such as pokeweed mitogen, could not be induced to switch to IgG or IgA production (*5*). Other reports suggest that the

R. C. Allen, D. K. Simoneaux, J. Belmont, Howard Hughes Medical Institute, Institute for Molecular Genetics, Baylor College of Medicine, Houston, TX 77030.
R. J. Armitage and W. C. Fanslow, Department of Immunology, Immunex Research and Development Corporation, Seattle, WA 98101.
M. E. Conley, Department of Pediatrics, University of Tennessee, College of Medicine and Department of Immunology, St. Jude Children's Research Hospital, Memphis, TN 38105.
H. Rosenblatt, Department of Pediatrics, Baylor College of Medicine, Houston, TX 77030.
N. A. Jenkins, N. G. Copeland, M. A. Bedell, Mammalian Genetics Laboratory, ABL–Basic Research Program, National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, MD 21702.
S. Edelhoff and C. M. Disteche, Department of Pathology, University of Washington, Seattle, WA 98195.
M. K. Spriggs, Department of Molecular Biology, Immunex Research and Development Corporation, Seattle, WA 98101.

*To whom correspondence should be addressed.