HUMAN GENE THERAPY 10:1885–1891 (July 20, 1999)
Mary Ann Liebert, Inc.

Brief Report

Purification of Recombinant Adeno-Associated Virus by Iodixanol Gradient Ultracentrifugation Allows Rapid and Reproducible Preparation of Vector Stocks for Gene Transfer in the Nervous System

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ABSTRACT

Recombinant adeno-associated virus (rAAV) vectors have become attractive tools for *in vivo* gene transfer. The production and purification of high-titer rAAV vector stocks for experimental and therapeutic gene transfer continue to undergo improvement. Standard rAAV vector purification protocols include the purification of the vector by cesium chloride (CsCl)-density gradient centrifugation followed by extensive desalination via dialysis against a physiological buffer for *in vivo* use. These procedures are extremely time consuming and frequently result in a substantial loss of the infectious vector titer. As an alternative to CsCl we have investigated the use of Iodixanol, an X-ray contrast solution, as the density-gradient medium. Purification of rAAV vectors by Iodixanol shortened the centrifugation period to 3 hr and resulted in reproducible concentration and purification of rAAV-vector stocks. We show that injection of rAAV derived from an Iodixanol gradient can be used for *in vivo* gene transfer applications in the brain and spinal cord without detectable cytopathic effects and directing stable transgene expression for at least 2 months.

INTRODUCTION

THE FIRST STUDY that demonstrated the feasibility of the use of adeno-associated virus (AAV) vectors for long-term gene transfer in the brain was published in 1994 (Kaplitt *et al.*, 1994). The AAV vector stocks used in that study were produced according to the original three-component AAV packaging system, consisting of a plasmid vector, a packaging plasmid, and adenovirus (Ad) as helper virus for recombinant AAV (rAAV) production (Samulski *et al.*, 1989). The viral vector was purified and concentrated by centrifugation of the crude cell lysate containing the vector over a 25% sucrose cushion. This resulted in relatively low-titer rAAV stocks, while absolute purification of the vector from adenovirus and serum proteins was not achieved. Considerable effort was invested in developing protocols to purify rAAV by CsCl density centrifugation (Bartlett *et al.*, 1996; Snyder *et al.*, 1996), which resulted in cleaner rAAV stocks, although contamination of the rAAV stocks with adenovirus could not be avoided. Moreover, the large-scale production of rAAV and its purification by equilibrium density-gradient centrifugation are elaborate and time consuming, necessitating the development of improved packaging plasmids. These studies mainly focused on finding a beneficial balance between *rep* and *cap* gene expression from the packaging plasmid as a means of obtaining optimal rAAV production (Flotte *et al.*, 1995; Li *et al.*, 1997; Vincent *et al.*, 1997; Grimm *et al.*, 1998). Subsequently, another modification of the original packaging system was introduced. In contrast to su-

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perinfecting the AAV producer cells with adenovirus, the essential adenovirus genes were provided on a helper plasmid. As a result of this, rAAV can be produced by a triple transfection of vector plasmid, packaging plasmid, and adenovirus helper plasmid (Xiao and Samulski, 1998).

A two-component AAV packaging system has been developed. This system requires the cotransfection of a vector plasmid and a hybrid plasmid containing the AAV genes and the essential Ad genes for rAAV vector production (Grimm *et al*., 1998). The use of this packaging plasmid results in high-titer rAAV vector stocks without the production of detectable wild-type AAV or adenovirus, thus makes the purification of rAAV from adenovirus superfluous.

Although the production of high amounts of rAAV per cell has been achieved, purification and concentration are still subject to improvement. We and others have found that the purification of high yields of rAAV by CsCl density-gradient centrifugation followed by dialysis of the rAAV-containing solution frequently results in a dramatic loss of the infectious rAAV titer. So far, only two publications have shown alternative approaches to purification and concentration of rAAV. The first report describes the reversible binding of rAAV to a sulfonated cellulose column (Tamayose *et al*., 1996). The second publication involves the reversible binding of rAAV to an affinity column coated with monoclonal antibodies specific to the AAV2 capsid (Grimm *et al*., 1998). However, a disadvantage of these methods is that elution of rAAV from the affinity column occurs in nonphysiological solutions. Since the adult brain contains nonmitotic neurons that are extremely sensitive to physiological changes the eluate, as such, cannot directly be used for gene transfer within the brain. We have now developed a rapid and reproducible method to purify and concentrate rAAV under physiological conditions for direct *in vivo* gene transfer. This method is based on the use of Iodixanol as a density gradient medium (Ford *et al*., 1994). Iodixanol {5,5′-(2-hydroxy-1,3-propanediyl)-bis(acetylimino)]bis-[*N,N′*-bis(2,3-dihydroxypropyl)-2,4,6-triiodo-1,3-benzenedicarboxamide]} is a nonionic, dimeric X-ray contrast medium with a significant margin of safety for neural tissue (Larsen *et al*., 1995). Because of its molecular mass and high solubility (1.32 g/ml) it can readily form self-generating isosmotic gradients whose profiles can be modulated by alterations to run parameters and starting concentration (Ford *et al*., 1994). The current protocol results in reproducible rAAV purification and concentration and can be performed in less than 1 day.

MATERIALS AND METHODS

Plasmids

pACVlacZ was constructed by inserting a CMV-lacZ expression cassette between the inverted terminal repeats (ITRs) of pSSV9 (Samulski *et al*., 1989). The 1.8-kb neuron-specific enolase (NSE) promoter (a gift from G. Sutcliffe, Scripps Clinic, La Jolla, CA) was cloned in pcDNA I/Amp (InVitrogen, San Diego, CA), yielding the plasmid pc5-NSE. The neurotrophin 3 (NT-3) cDNA, isolated from pc5-NT-3 (Dijkhuizen *et al*., 1997), was cloned downstream of the NSE promoter of pc5-NSE. Similarly, the "humanized" green fluorescent protein (GFP) cDNA (Crameri *et al*., 1996) was isolated from pc5-GFP and cloned in pc5-NSE. The brain-derived neurotrophic factor (BDNF) cDNA was cloned into pcDNA I/AMP. Subsequently, pTR-CMVnt-3, pTR-NSEnt-3, pTR-CMVbdnf, and pTR-NSEgfp were constructed by inserting the corresponding transgene expression cassettes between the AAV-ITRs of pTR-UF (Zolotukhin *et al*., 1996). The construction of the helper plasmid pDG has been described previously (Grimm *et al*., 1998).

Viral vector production

Ten culture dishes (100-mm diameter), each containing 5×10^6 293T cells, were cotransfected by calcium phosphate with a total of 30 μg of the vector plasmid and packaging plasmid (pDG) at a 1:1 molar ratio. After 6 hr the transfection medium was replaced by fresh culture medium and the cells were incubated at 48 hr at 37°C and 5% CO_2.

Viral vector purification and concentration

Harvesting. The cells were dislodged from the culture dishes and pelleted by centrifugation at $500 \times g$. The cells were then resuspended in 20 ml of 10 m*M* Tris-HCl–1 m*M* $MgCl_2$ (pH 8.0), freeze–thawed three times, and treated with DNase I (10 μg/ml) for 30 min at 37°C. The cell debris was spun down at $3000 \times g$ for 20 min at 4°C. Next, ammonium sulfate precipitation or column chromatography was used to concentrate the virus.

Ammonium sulfate precipitation. A one-third volume of saturated $(NH_4)_2SO_4$ was added dropwise to the supernatant at 4°C and left on ice for 10 min. The precipitate was centrifuged at $5000 \times g$ for 20 min at 4°C. Two-thirds of the starting volume of saturated $(NH_4)_2SO_4$ was added dropwise to the supernatant and left for 20 min on ice. The precipitate was centrifuged at $10{,}000 \times g$ for 20 min at 4°C.

Cellufine sulfate column chromatography. The supernatant containing the virus was loaded onto a 3-ml column of Matrex Cellufine sulfate bead medium (Amicon, Danvers, MA). After the supernatant had run through, the column was washed with 20 ml of phosphate-buffered saline (PBS, pH 7.2). Next, the virus was eluted from the column with 3 ml of PBS containing 1 *M* NaCl, pH 7.2.

After either of these procedures an optimized CsCl purification protocol (Bartlett *et al*., 1996) or the Iodixanol purification protocol was used.

CsCl protocol. After ammonium sulfate precipitation the pellet containing the virus was dissolved in 5 ml of CsCl solution (density, 1.37 g/ml) and centrifuged in an SW55 Ti rotor (Beckman, Fullerton, CA) at 35,000 rpm for 36 hr at 16°C. After centrifugation, gradient fractions were collected from the bottom of the tube and dialyzed three times against 1 liter of PBS, pH 7.5.

Iodixanol protocol. After ammonium sulfate precipitation the pellet containing the virus was dissolved in 2.7 ml of PBS, pH 7.4, and transferred to a 5-ml Quick-Seal (Beckman Instruments,

CA) ultracentrifuge tube. After column chromatography the eluate (2.7 ml) was transferred to a 5-ml Quick-Seal ultracentrifuge tube. In both cases the virus solution was underlaid with an equal volume of Optiprep (a 60% solution of Iodixanol in water, purchased from Nycomed Pharma AS, Oslo, Norway). The tube was sealed and a gradient was performed by rotating the tube around its axis for 12 min at 20 rpm at an angle of 80° in a test tube rotator (Snijders Scientific, The Netherlands). The tube was placed into a NVT90 rotor (Beckman Instruments) and centrifuged at 71,0000 rpm for 3 hr at 16°C. Fractions of approximately 300 μl were collected from the bottom of the gradient. The rAAV titers were determined by one of the assays described below. For application within the nervous system, rAAV derived from the Iodixanol gradient was diluted 10 times with PBS, pH 7.5, to reduce the viscosity of the Iodixanol, and was subsequently reconcentrated by centrifugation in a Centricon100 concentrator (Amicon).

Viral vector titering assays

The transgene expressing unit titer (TU/ml) was determined by coinfection of 293 cells with dilutions of the viral vector and adenovirus (multiplicity of infection [MOI] of 3). After 24 hr, transgene-expressing cells were visualized by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) histochemistry or in the case of rAAV-CMVbdnf by *in situ* hybridization with a digoxigenin (DIG)-labeled riboprobe (Boehringer Mannheim, Germany).

The infectious unit titer (ICU/ml) was determined by an infectious center assay (Salvetti *et al*., 1998). Briefly, dilutions of the vector, and adenovirus (MOI of 10), were coinfected on HeLaRC32 cells that stably express the AAV *rep* and *cap* genes (Salvetti *et al*., 1998). After 24 hr the cells were blotted onto a GeneScreen nylon membrane. Vector DNA was detected with DIG-labeled riboprobes (Boehringer Mannheim) and visualized by immunochemoluminescence exposure to X-Omat film (Kodak, Rochester, NY).

The vector particle titer (VP/ml) was determined by treating vector dilutions with DNase I (10 μg/ml) for 30 min, followed by digestion with proteinase K (2 mg/ml)–0.6% sodium dodecyl sulfate (SDS)–10 m*M* EDTA for 1 hr. After phenol–chloroform extraction vector DNA was precipitated and the pellet was dissolved in 0.4 *M* NaOH–10 m*M* EDTA. Dot-blot

TABLE 1. rAAV VECTOR STOCK PREPARATIONS BASED ON THE USE OF CsCl OR IODIXANOL[a]

Vector	*Prec. or column*	*Gradient medium*	*TU/ml in gradient medium* (a)	*TU/ml after dialyses or centricon* (b)	*VP/ml after dialyses or Centricon* (c)	*Recovery (%)* (b/a)	*Ratio VP/TU or VP/ICU* (c/b)	*Final stock volume (ml)*
AAV CMVlacZ	Prec.	CsCl	1.1×10^8	$<10^3$	ND	<0.001	ND	0.5
		CsCl	4.0×10^8	2.0×10^3	ND	0.0005	ND	0.5
		CsCl	3.8×10^8	4.5×10^7	ND	12	ND	ND
		CsCl	6.0×10^8	$<10^3$	ND	<0.002	ND	ND
		CsCl	2.0×10^9	4.0×10^3	5.0×10^{11}	0.0002	1.3×10^8	0.5
		CsCl	3.1×10^8	1.7×10^8	2.0×10^{11}	55	1176	0.4
		CsCl	1.6×10^9	1.0×10^9	2.5×10^{11}	61	250	0.3
		CsCl	1.2×10^8	6.6×10^7	1.6×10^{11}	55	2424	0.7
		CsCl	1.8×10^9	5.3×10^4	5.0×10^{11}	0.003	9.4×10^6	0.5
	Prec.	Iodixanol	2.5×10^9	2.4×10^9	1.6×10^{12}	96	66	0.1
		Iodixanol	4.3×10^8	1.0×10^8	5.9×10^{10}	23	585	0.1
		Iodixanol	3.1×10^9	6.8×10^8	8.2×10^{11}	22	1200	0.2
		Iodixanol	1.2×10^9	1.5×10^9	1.6×10^{12}	121	1081	0.1
		Iodixanol	2.5×10^9	1.7×10^9	6.8×10^{11}	68	400	0.3
		Iodixanol	4.3×10^8	3.2×10^8	1.2×10^{11}	74	385	0.3
AAV CMVbdnf		Iodixanol	8.6×10^7	1.0×10^8	1.0×10^{11}	116	1052	0.4
AAV CMVnt-3		Iodixanol	2.3×10^9	2.0×10^{9b}	6.6×10^{11}	85	329	0.2
AAV CMVbdnf		Iodixanol	7.7×10^8	2.0×10^8	7.3×10^{10}	26	364	0.3
AAV NSEgfp		Iodixanol	2.1×10^9	2.0×10^{9b}	1.5×10^{12}	96	727	0.3
AAV NSEnt-3		Iodixanol	1.9×10^9	1.5×10^{9b}	2.0×10^{12}	80	1333	0.3
AAV CMVlacZ	Column	Iodixanol	3.3×10^{10}	8.3×10^9	3.2×10^{12}	25	385	0.3
		Iodixanol	2.2×10^9	2.0×10^9	1.2×10^{12}	91	600	0.3
		Iodixanol	4.6×10^9	4.0×10^9	2.0×10^{12}	87	500	0.3

Abbreviation: ND, Not done

[a]Comparison of rAAV purified by either CsCl or Iodixanol density centrifugation after virus precipitation with ammonium sulfate (prec.) or column chromatography (column). Shown here is the percentage of recovery of AAV vector after dialysis of the CsCl gradient fractions or after Centricon 100 treatment of the Iodixanol gradient fractions. In CsCl gradients as well as in Iodixanol gradients similar high titers of rAAV were obtained (*a*). However, in the dialysis of CsCl gradient fractions an extremely low recovery was observed in five of nine experiments (*b*). In none of the experiments performed with Iodixanol was a dramatic decline in infectious vector titer observed during vector stock preparation (*b*). The data shown here represent the average titer determined by assays performed in duplicate.

[b]ICU/ml.

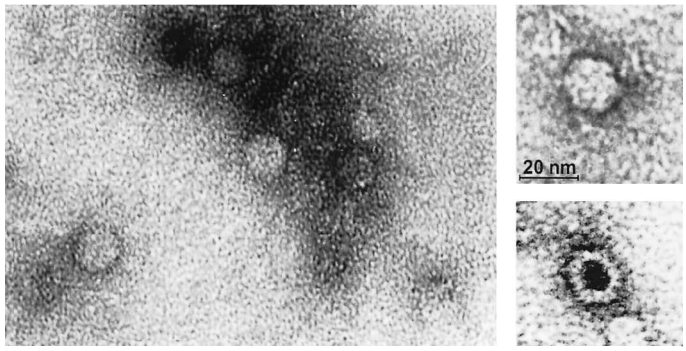

FIG. 1. Viral vector particles purified by Iodixanol gradient are visualized by electron microscopy by negative staining with uranyl acetate. *Right:* Magnification of individual particles: top, intact full virus particle; bottom, empty virus particle. Scale bar: 20 nm.

hybridization was performed using a Hybri-Dot manifold assembly (Life Technologies, Gaithersburg, MD). Vector DNA was detected as described above.

In vivo *infections of viral vector stocks*

Brainstem injections (Hermens and Verhaagen, 1997) were performed with 1 μl of rAAV-CMVlacZ containing 3×10^5 TU in 60% Iodixanol–PBS, or in 6% Iodixanol–PBS. Injections to the spinal cord (Zhang *et al*., 1998) were performed with 2 μl of rAAV-NSEgfp containing 4×10^6 ICU in 6% Iodixanol–PBS.

Immunohistochemistry

After anesthesia with sodium pentobarbital (Nembutal), rats injected in the brainstem were sacrificed 7 days postinjection by perfusion with 4% paraformaldehyde. Cryostat sections (50 μm) were processed for β-galactosidase (β-Gal) immunohistochemistry to detect the transgene expression, or processed for ED-1 antigen expression to detect macrophages and microglia (Hermens and Verhaagen, 1997). Rats injected in the spinal cord were sacrificed 2 months postinjection by perfusion with 4% paraformaldehyde. Cryostat sections (50 μm) were processed for green fluorescent protein immunohistochemistry as described previously (Peel *et al*., 1997).

Protein gel electrophoresis

Ten microliters of viral vector stocks (containing 2.0×10^9, 2.5×10^9, 7.3×10^8, 1.6×10^{10}, 1.2×10^{10}, and 2.0×10^{10} VP was evaluated for cellular protein content by electrophoresis in 8% polyacrylamide gels. The proteins in the gel were stained overnight with Coomassie Brilliant Blue G250 and the gel was destained with a solution containing 25% (v/v) methanol–70% (v/v) glacial acid.

Electron microscopy

An aliquot of viral vector stock solution was loaded onto carbon-coated 150 mesh nickel grids for 2 min and stained for 3 min with 2% uranyl acetate.

RESULTS AND DISCUSSION

The production of large amounts of rAAV vector particles free of contaminating wild-type AAV and adenovirus has long been difficult to achieve for vectorologists. Several new approaches have improved the production of rAAV (Li *et al*., 1997; Xiao and Samulski, 1998; Grimm *et al*., 1998). For this study we used an approach that requires only the cotransfections of the vector plasmid and a hybrid AAV/Ad plasmid

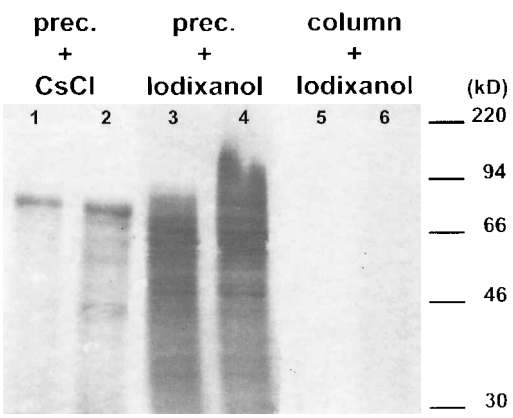

FIG. 2. Protein gel electrophoresis of rAAV samples purified by either ammonium sulfate precipitation (prec.) followed by a CsCl gradient (lanes 1 and 2), precipitation followed by Iodixanol gradient (lanes 3 and 4), and Cellufine sulfate column chromatography (column) followed by Iodixanol gradient (lanes 5 and 6). Proteins are detectable in vector preparations including ammonium sulfate precipitation. However, vector preparations using Cellufine sulfate chromatography do not contain detectable proteins.

(pDG) to obtain high yields of rAAV free of adenovirus (Grimm *et al*., 1998).

Purification of rAAV by cesium chloride density-gradient centrifugation

Purification and concentration of rAAV in a CsCl gradient frequently resulted in the loss of infectious vector titer (Table 1). To determine at which stage of rAAV stock preparation the infectious titer decreased, we determined the titer after several steps of the purification protocol. From these data we observed that there were two critical steps that led to a decrease in the infectious rAAV titer. Frequently, the mere storage at 4°C of high-titer rAAV samples derived from a CsCl gradient resulted in a decrease in the infectious viral vector titer of several orders of magnitude. On the other hand, storage at 16°C for up to 1 week never resulted in a significant decrease in infectivity of rAAV-containing CsCl gradient fractions (data not shown).

The second cause of the decline in rAAV titer occurred during the desalination of rAAV samples derived from the CsCl gradient. Since CsCl is toxic to cells, CsCl-containing rAAV samples should be extensively desalinated before such samples can be used for *in vivo* transduction experiments. Desalination is usually achieved by dialysis. On many occasions (five of nine experiments; Table 1), however, dialysis of rAAV samples resulted in a decrease in infectious vector titer of several orders of magnitude. In contrast, dot-blot titers of rAAV samples remained on the order of 10^{11} particles/ml (Table 1), excluding the possibility that the loss of infectious titer was due to adherence of vector particles to the dialysis membrane. We suggest that during storage at 4°C or dialysis viral vector particles may aggregate, resulting in a loss of infectivity. The occurrence

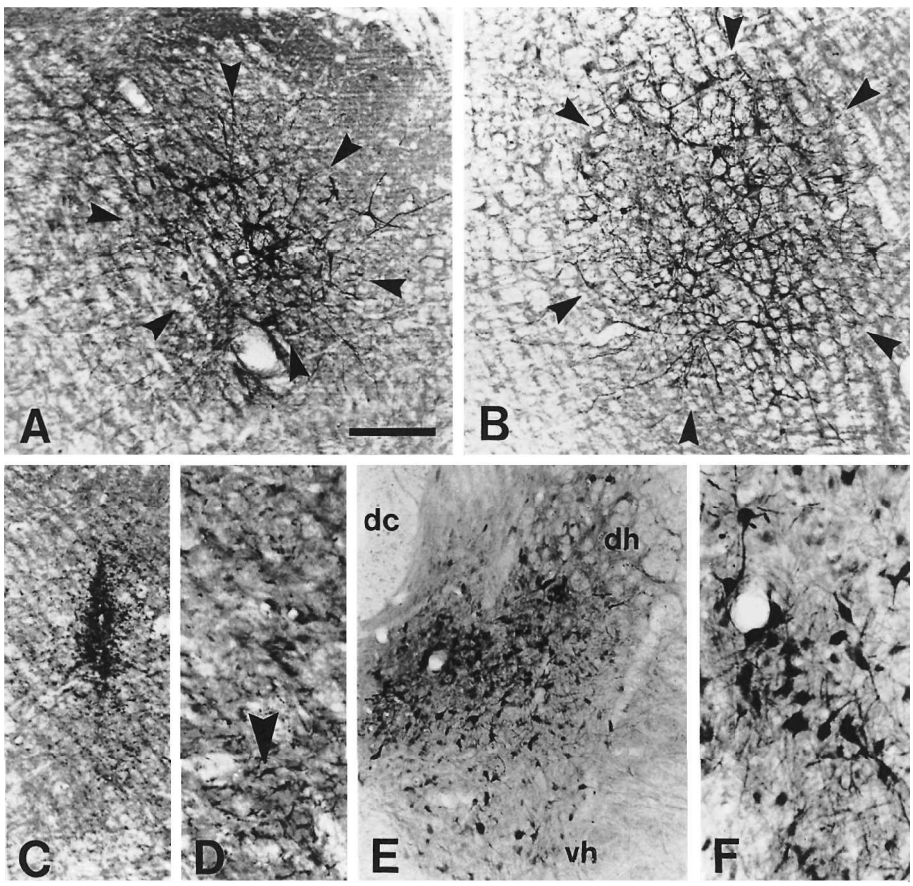

FIG. 3. The performance of AAV vector purified by ammonium sulfate precipitation followed by Iodixanol gradient centrifugation in the brain and spinal cord of the rat. Injection of 1 μl of rAAVlacZ derived from the Iodixanol gradient containing 3×10^5 TU in the parenchyma of the brainstem (**A**). The 50-μm-thick section shows transduced neurons 7 days postinjection. The spread of the vector through the parenchyma [see arrowheads in (**A**)] is less compared with the spread of the vector in PBS [arrowheads in (**B**)]. Injection of 1 μl of rAAVlacZ in 60% Iodixanol–PBS in the parenchyma does not result in signs of toxicity (**A**). ED1-positive macrophages are observed only in the needle track (**C**). ED1-positive microglial cells observed in the vicinity of the needle track have a ramified appearance, indicating that these cells are "resting" microglia [arrowhead in (**D**)]. Injection of 2 μl of rAAVNSEgfp (4×10^6 ICU) in the dorsal horn of the spinal cord results in the transduction of many neurons 2 months after administration of the vector (**E**). The magnification of the section in (**E**) shows that multiple neurons transport the transgene in their processes, and that no toxicity can be observed (**F**). dh, Dorsal horn; vh, ventral horn; dc, dorsal column. Scale bar: (**A, B, C,** and **E**) 314 μm; (**D** and **F**) 125 μm.

of irreversible aggregation of empty AAV capsids during dialysis has been described previously (Steinbach *et al*., 1997).

Purification of rAAV by Iodixanol density gradient centrifugation

The use of Iodixanol allowed the separation of rAAV from much of the cellular material and the concentration of rAAV in a small fraction of the gradient within 3 hr of ultracentrifugation. After centrifugation the upper part of the gradient displayed a cloudy white appearance consistent with cellular debris, which was demarcated by two thin, sharp bands located one-third the distance from the bottom of the tube and presumably represententing cell organelles (Ford *et al*., 1994). AAV vector was detected in fractions 0.5 to 1.0 ml below the observed bands. At the bottom of the gradient, fractions were extremely viscous, but the fractions containing the vector displayed less viscosity (density of ~1.3 g/ml; refractive index of 1.42; 60% Iodixanol). The reduce the viscosity further the rAAV-containing fraction was diluted 10-fold with PBS, followed by reconcentration of the rAAV with a Centricon100 concentrator. This procedure reduced the Iodixanol concentration of the rAAV-containing fraction to approximately 6%, with an average recovery of 73% (Table 1). Detection by electron microscopy of rAAV after the Iodixanol gradient showed that the purified rAAV particles were intact. A mixture of full and empty vector particles could be observed (Fig. 1).

Since the purity of rAAV vectors is critical for preclinical and phase I clinical studies we compared the protein content of rAAV–vector samples purified by CsCl and Iodixanol by protein gel electrophoresis. We observed a smear of protein in lanes containing rAAV purified in CsCl as well as in Iodixanol gradients (Fig. 2, lanes 1 to 4). However, ammonium sulfate-precipitated vector particles purified by an Iodixanol gradient were more contaminated with cellular proteins (Fig. 2, lanes 3 and 4). Therefore, instead of ammonium sulfate precipitation of the virus, we introduced a Cellufine sulfate column chromatography step (Tamayose *et al*., 1996) to purify rAAV and combined this technique with the Iodixanol gradient to concentrate the viral vector. In this protocol the column eluate containing the vector was loaded onto an equal volume of Optiprep and centrifuged. This combination resulted in rAAV stocks that did not contain detectable cellular proteins as judged by Coomassie blue-stained protein gels (Fig. 2, lanes 5 and 6).

Infusion of infectious rAAV derived from the Iodixanol gradient

Since we could show that rAAV purified by Iodixanol remained infectious for cells in culture, the next step was to study the infectivity of rAAV derived from the Iodixanol gradient *in vivo*. Injection of 1 μl of rAAVlacZ, directly obtained from the Iodixanol gradient containing 3×10^5 TU, resulted in the transduction of many neurons after administration of the vector within the brainstem parenchyma (Fig. 3A). However, probably owing to the viscosity of the Iodixanol in the fraction, the spread and therefore the distance of transduced neurons from the needle track appeared to be restricted. A reduction in the viscosity of the Iodixanol-containing rAAV fraction by use of a Centricon100 concentrator resulted in a larger area of transduced cells in the brain parenchyma (Fig. 3B).

The application of Iodixanol as an inoculum for *in vivo* gene transfer had not been reported thus far, to our knowledge. Therefore, it was necessary to determine whether Iodixanol had an adverse effect on brain tissue. No difference in both cytoarchitecture or the presence of macrophages were observed in rats injected with rAAV in either 60% Iodixanol–PBS (i.e., gradient fraction) or in 6% Iodixanol–PBS (i.e., Centricon100 fraction). ED1-positive macrophages were observed in and around the needle track 1 week postinjection of the vector (Fig. 3C). The majority of ED1-positive cells in the vicinity of the needle track exhibited a ramified appearance, indicating the presence of "resting" microglial cells (Fig. 3D). No cytotoxicity beyond the needle track was observed. In addition, long-term expression of green fluorescent protein (GFP) was observed after injection of 4×10^6 ICU of rAAV-NSEgfp in the spinal cord. Two months after injection of the vector multiple neurons in the spinal cord gray matter were transduced without notable cytotoxicity (Fig. 3E and F).

In conclusion, we report here the use of Iodixanol as a density-gradient medium to purify and concentration rAAV rapidly and in a reproducible manner, thus solving the problem of loss of infectious rAAV titers during dialysis after standard CsCl gradient protocols and substantially reducing the time required to prepare vector stocks. The rAAV samples prepared by ammonium sulfate precipitation of the virus followed by Iodixanol density-gradient centrifugation evinced no cytotoxicity or an inflammatory response other than previously observed after injection of CsCl-purified rAAV samples. Furthermore, long-term transgene expression is observed in neurons in the brainstem and spinal cord, suggesting a suitable working protocol for animal gene transfer applications. However, we emphasize that the preliminary studies presented here, including the column chromatography technique prior to the Iodixanol gradient in order to avoid injection of contaminating proteins derived from the producer cells, should be explored further for gene therapy applications in species other than rodents. With respect to this, we suggest that the pure rAAV samples obtained by an AAV-specific affinity column chromatography technique (Grimm *et al*., 1998) may be combined with the currently presented Iodixanol gradient technique to optimize further the protocol for therapeutic rAAV-mediated gene transfer in primates.

ACKNOWLEDGMENTS

We thank Dr. P. Moullier for providing us with the HeLaRC32 cell line, and Dr. J.G. Sutcliffe for his gift of the NSE promoter. We thank Dr. Bob Baker for critically reading the manuscript, and Gerben van der Meulen for assistance with the photography. This work was funded by The Netherlands Organization for Scientific Research (NWO).

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Received for publication October 12, 1998; accepted after revision May 7, 1999.