Vol. 213, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 4, 1995 Pages 81-87

# UPTAKE OF EXOGENOUS DNA BY RAT LIVER: EFFECT OF CATIONIC LIPIDS

Robert WATTIAUX, Michel JADOT, Franz DUBOIS, Sandra MISQUITH*
and Simone WATTIAUX-DE CONINCK

Laboratoire de Chimie Physiologique, Facultés Universitaires Notre- Dame de la Paix, 61, rue de Bruxelles, B. 5000 Namur, Belgium

* St Joseph's College, P.B. 5031, Residency Road, Bangalore 560 001, India

Received June 13, 1995

We have investigated by using centrifugation methods, the uptake and the intracellular fate of $^{35}S$ DNA by rat liver and the effect on these processes of N-(1-(2,3-dioleoxyloxy)propyl)-N,N,N,-trimethylammonium-methyl-sulfate(DOTAP, Boehringer, Mannheim, Germany), an artificial cationic lipid frequently used in transfection experiments. Labeled DNA molecules are quickly taken up by the liver but a progressive degradation takes place with time. Subcellular distribution of the radioactivity was established after differential and isopycnic centrifugation. Results indicate that $^{35}S$ DNA enters liver cells by endocytosis and reaches lysosomes. The uptake of $^{35}S$ DNA is not modified if the molecule is associated with DOTAP but marked differences are observed after internalization of the macromolecule. When DOTAP is used, radioactive products remain for a long time in low density organelles distinct from lysosomes indicating that the transfer of internalized DNA to these organelles is delayed by the cationic lipid.These results suggest that cationic lipids could favor transfection by preventing the delivery of DNA to lysosomes, allowing these molecules to be kept intact and available for transfer from endosomes to cytosol for a long time.


Cell transfection "ex vivo" of genetic material and introduction of oligonucleotides into cells are widely used in cellular and molecular biology research. More recently, transfection "in vivo" by direct injection of animals with nucleic acid molecules has been performed with success. Nucleic acids and oligonucleotides are large hydrophilic molecules that normally can not cross biological membranes. Some observations on cultured cells suggest that the uptake of these molecules occurs by endocytosis(1,2). Addition of a cationic lipid to nucleic molecules considerably increases efficiency of the transfection (3) by a mechanism not yet elucidated. It has been proposed that the beneficial effect of these lipids could originate from the fact that these molecules facilitate the transfer of the nucleic compounds through the plasma or the endosomal membrane (1,3). Moreover, they could make nucleic acids more resistant to nucleases(2). The aim of our investigations was to obtain information by using centrifugation methods on: 1) the uptake and the intracellular fate of exogenous nucleic molecules "in vivo", 2) the effect of cationic lipids on these processes. Our research was first focused on the liver as it is easy to apply centrifugation techniques

0006-291X/95 $12.00

to that organ. In addition, recently, it has been reported that succesfull transfection of liver with genetic material was possible (4,5) even after intravenous injection of DNA(6). Our results indicate that DNA associated or not with a cationic lipid enters the liver by endocytosis but that the cationic lipid markedly delays the transfer of the nucleic molecules to lysosomes.

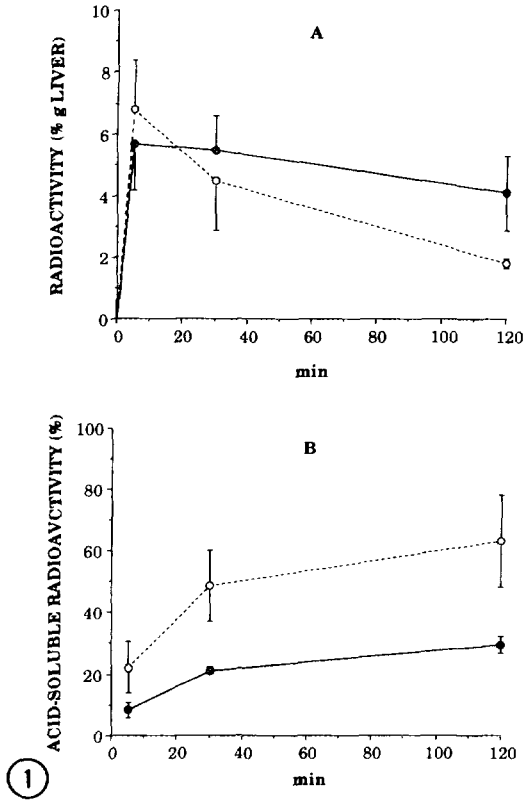
## MATERIALS AND METHODS

Experiments were performed with male Wistar rats weighing 300-350 g. Labeled DNA was obtained by nick translation of pcDNAI/Amp plasmid (Invitrogen, Abingdon, U.K.) with $^{35}S$ dATP( kit 5500, Amersham, Buckinghamshire, U.K.); analysis by agarose electrophoresis and by chromatography on Sephacryl S- 500 shows that the labeled product is mainly composed of 500 KD nucleic acid molecules. Rats were injected intravenously with 0.6 ml of 0.15 M NaCl containing 100 ng of $^{35}S$ DNA with or without 500 ng of an artificial cationic lipid N-(1-(2,3-dioleoyloxy)propyl)-N,N,N,-trimethylammonium-methyl-sulfate(DOTAP,Boehringer,Mannheim, Germany) and killed at various times after injection. The liver was perfused with cold 0.15 M NaCl, removed and homogenized in ice-cold 0.25 M sucrose. Differential centrifugation was performed according to de Duve et al.(7) and isopycnic centrifugation according to Beaufay et al.(8). Cathepsin C was measured as described by Jadot et al.(9) and arylsulfatase by the method of Bowers et al.(10). Degradation of $^{35}S$ DNA after its uptake, was assessed by measuring the acid-soluble radioactivity in 5% perchloric acid.

## RESULTS

### *$^{35}S$ DNA in absence of DOTAP*

As illustrated in Fig 1A, labeled DNA molecules are quickly taken up by the liver. With time, total radioactivity decreases when the percentage of acid-soluble radioactivity increases, indicating a progressive degradation of the nucleic acid molecules. Distribution after differential centrifugation was established at different times after injection. A nuclear fraction N, a heavy mitochondrial fraction M, a light mitochondrial fraction L, a microsomal fraction P and a soluble fraction S were isolated according to the scheme of de Duve et al (7). The percentages of radioactivity recovered in the fractions are given in Fig 2A. Early after injection, radioactivity is shared between the mitochondrial fractions M and L and the microsomal fraction P. With time, the amount of radioactivity associated with P decreased to the benefit of the mitochondrial fractions, moreover the proportion located in the unsedimentable fraction S increases.It is to be noted that radioactivity present in S is mostly acid-soluble.Total mitochondrial fractions (M+L) were analyzed by isopycnic centrifugation at increasing times after injection. The distributions of radioactivity and of a lysosomal marker cathepsin C are illustrated on Fig 3A. At any time, radioactivity is recovered in the regions of the gradient where cathepsin C is located. Such results suggest that internalized DNA is quickly transferred to lysosomes. A typical characteristic of liver lysosomes is that their density is markedly lowered after the animal is injected with Triton WR 1339 a non ionic detergent, as a result of



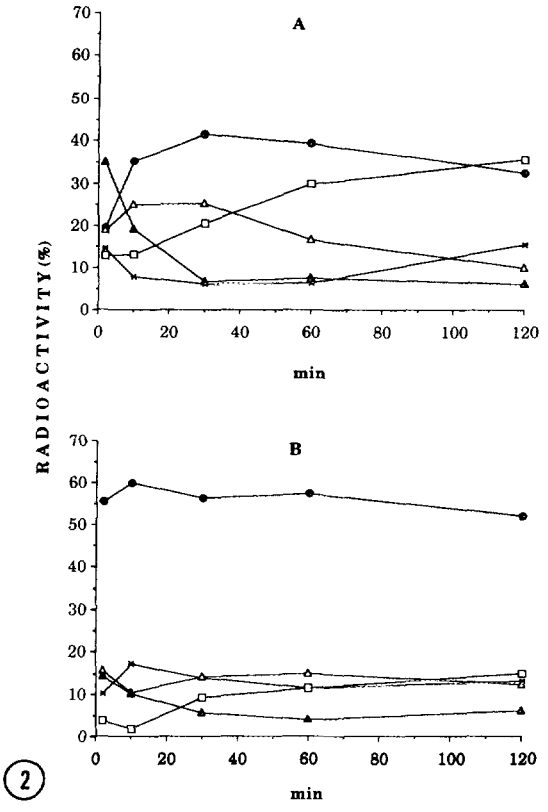




FIG. 1.
*Uptake of $^{35}S$ DNA by rat liver.* Radioactivity was measured in homogenates of rat liver at increasing times after injection of $^{35}S$ DNA without (○--○) or with (●–●) DOTAP. (A) Total radioactivity, the values are given as percentages of the injected dose/g liver. (B) Acid-soluble radioactivity, the values are given as percentages of the total radioactivity . Means for three animals with SD are presented.

FIG. 2.
*Distribution of radioactivity after differential centrifugation by the method of de Duve et al.(7).* The radioactivity distributions were obtained with livers of rats killed at increasing times after injection of $^{35}S$ DNA without (A) or with (B) DOTAP. The values are given as percentages of the radioactivity recovered in homogenates. N(X), nuclear fraction; M(●), heavy mitochondrial fraction; L(△), light mitochondrial fraction; P(▲), microsomal fraction; S(□), soluble fraction.

accumulation of that substance in these organelles(11). We have investigated the effect of the detergent on structures with which radioactivity is associated after $^{35}S$ DNA injection. Results are presented in Fig 4. After 2 min, Triton WR 1339 does not affect the distribution of radioactivity, showing that at that time labeled molecules are in a prelysosomal compartment. After 60 min, a shift of radioactivity distribution takes place towards lower density regions of the gradient as it is observed for cathepsin C and arylsulfatase, indicating that, at that time most of the radioactivity is located in lysosomes.

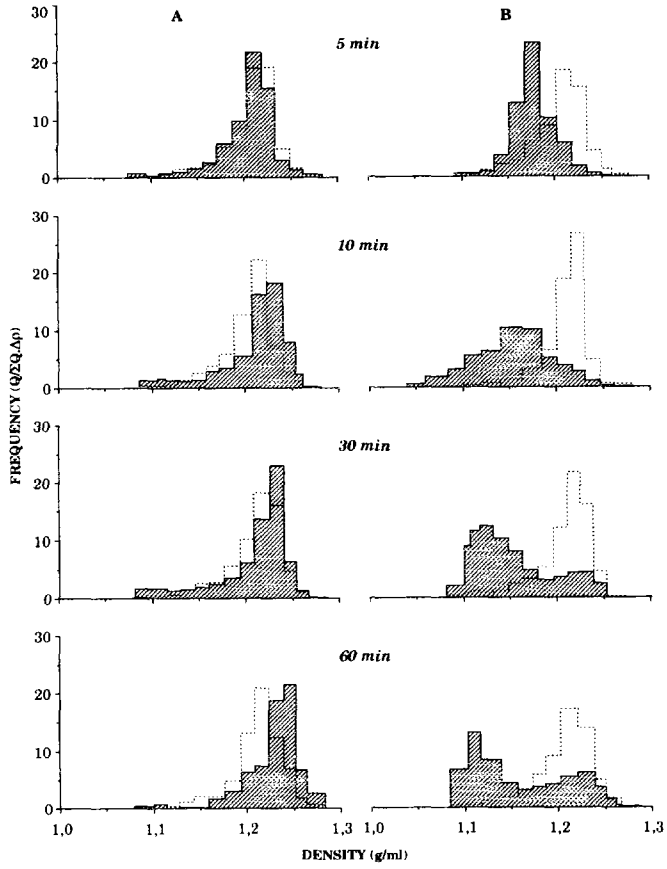



FIG. 3.
*Density distribution histograms of radioactivity and cathepsin C after isopycnic centrifugation of a total mitochondrial fraction (M+L) in a sucrose gradient.* The particle preparations were isolated at increasing times after $^{35}S$ DNA injection without (A) or with (B) DOTAP. Centrifugations were performed at 240,000 g in the VTI 65 Beckman rotor for 180 min. The sucrose gradient extended from 1.09 to 1.30 g/ml density. Ordinate: frequency $Q/\Sigma Q.\Delta\rho$ where Q represents the activity found in the fraction, $\Sigma Q$, the total activity recovered in the sum of the fractions and $\Delta\rho$, the increment of density from top to bottom of the fraction. Shaded area: radioactivity. Dotted line: cathepsin C.

*$^{35}S$ DNA in presence of DOTAP*

As shown in Fig 1B, the uptake of $^{35}S$ DNA by the liver is not significantly affected by the presence of DOTAP. However, the decrease of liver radioactivity and the increase of acid-soluble radioactivity that occur with time are markedly slower when DOTAP is used. Two significant differences are observed in differential centrifugation (Fig 2B): the cationic lipid brings about a very early association of radioactivity with the heavy mitochondrial fraction M and slows down the release of radioactivity in S. Striking differences in the distributions are observed after isopycnic centrifugation (Fig 3B). When DOTAP is used, up to at least 10 min, radioactivity distribution curve is unimodal but clearly distinct from that of the lysosomal enzyme, being shifted towards

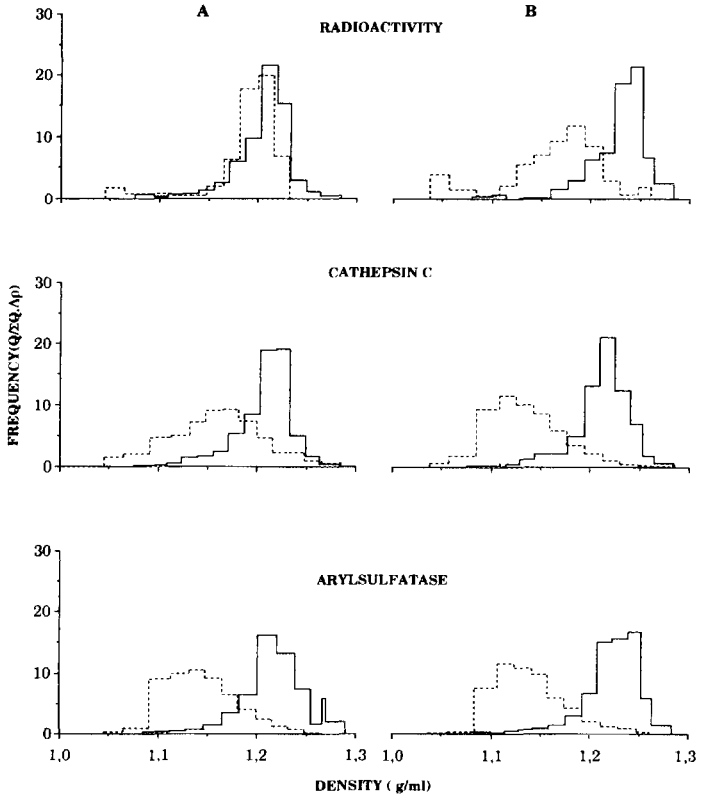



FIG. 4.
*Density distribution histograms of radioactivity, cathepsin C and arylsulfatase after isopycnic centrifugation of a total mitochondrial fraction (M+L) in a sucrose gradient. Effect of Triton WR 1339.* The mitochondrial fractions were isolated 2 min (A) or 60 min (B) after $^{35}S$ DNA injection; the animals were (- - - - ) or were not (——— ) injected with Triton WR 1339 (170 mg in 1 ml of 0.15 M NaCl) four days before receiving $^{35}S$ DNA injection.

low density zones. Later, the distribution of labeled molecules becomes bimodal, a peak of radioactivity becomes apparent in the fractions where cathepsin C is found but even after 60 min, a major peak is still located in the low density regions of the gradient. The distribution of lysosomes is not affected by DOTAP, as ascertained by cathepsin C distribution.

## DISCUSSION

Our results show that $^{35}S$ DNA is rapidly taken up by the liver and that degradation of the molecule is relatively quick as ascertained by the appearance of acid-soluble radioactivity in increasing amounts with time. Differential centrifugation illustrates that early after injection, radioactivity is shared between the large granule fraction(M+L) and the microsomal fraction(P) but with time, the proportion associated with P decreases while that found in M+L increases. It is the classical

picture observed for a substance endocytosed by the liver. It is explained by the progressive transfer of this compound from small vesicles: pinosomes to bigger ones: endosomes and lysosomes. This clearly indicates that the uptake of DNA by the liver involves an endocytic process. Although, no detailed kinetic analysis of the uptake was done in our work, our results allow us to suggest that DNA is taken up by an adsorptive or a receptor-mediated endocytosis; indeed, fluid phase endocytosis would be largely insufficient to explain the quick accumulation of radioactivity that we observe in the organ .

Isopycnic centrifugation shows that radioactivity associated with sedimentable components exhibits a distribution pattern in a sucrose gradient similar to that of lysosomal enzymes. However, the effect on these structures of Triton WR 1339, a specific lysosome density perturbant, shows that they are composed of two kinds of organelles. One, through which radioactive molecules travel first. It has a density that is not affected by the detergent and therefore, can be considered as distinct from lysosomes; another one in which radioactivity is later located, has a density that is decreased by Triton WR 1339 treatment and corresponds to lysosomes. Such a behaviour in isopycnic centrifugation of subcellular structures with which radioactivity is associated illustrates that the journey of endocytosed DNA involves a prelysosomal (endosomal) compartment before ending in lysosomes.

The uptake of DNA by the liver is not modified if the molecule is associated with the cationic lipid DOTAP. This shows that DOTAP does not interfere with endocytosis of the nucleic molecules at the level of the plasma membrane. On the other hand, considerable differences are observed after internalization of the macromolecule. As shown by isopycnic centrifugation, when DOTAP is used, radioactive products remain for a long time in low density organelles, well separated from lysosomes in sucrose gradient, the distribution of these latter organelles being not changed by DOTAP. Therefore, the transfer of DNA to lysosomes, the main intracellular site of degradation of an endocytosed substance, is put off when DOTAP is associated with the nucleic molecule, which probably explains why the production of acid-soluble labeled molecules is delayed in these conditions. The nature of these structures, that must correspond to endosomal organelles, is not known. Their size is relatively high since they mostly sediment like mitochondria, in the heavy mitochondrial fraction M . With respect to their low density, it could originate from the presence of DOTAP. Indeed, a complex made of DOTAP and DNA with a ponderal ratio of 5/1 as we made use has a density of about 1.09 g/ml; endosome like structures accumulating such a complex could be endowed with a very low density.

The mechanism by which cationic lipids increase efficiency of transfection is still unclear. Our observations bring a new insight into the question. They suggest that cationic lipids could favor transfection by delaying the delivery of endocytosed DNA to lysosomes, allowing these molecules to be kept intact and available for transfer from

endosomes to cytosol for a long time. Why association of DNA with DOTAP prevents the transfer of nucleic acid molecules to lysosomes is currently investigated.

ACKNOWLEDGMENT

This work was supported by the Fonds de la Recherche Scientifique Médicale (contract n° 3.4503.95).

REFERENCES

1- Zhou, H. and Huang, L. (1994) *Biochim. Biophys. Acta.* 1189, 195-203.
2- Capacioli, S., Di Pasquale, G., Mini, E., Mazzei, T. and Quattrone, A. (1993) *Biochem. Biophys. Res. Commun.* 197, 818-825.
3- Felgner, P.L., Gadek, T.R., Holm, M., Roman, R., Chan, H.W., Wenz, M., Northrop, J.P., Ringold, G.M. and Danielsen, M. (1987) *Proc. Natl. Acad. Sci. USA.* 84, 7413-7417.
4- Wu, G.Y., Wilson, J.M., Shalaby, F., Grossman, M., Shafritz, D.A. and Wu, C.H. (1991) *J. Biol. Chem.* 266, 14338-14342.
5- Wilson, J.M., Grossman, M., Wu, C.H., Roy Chowdhury, N., Wu, G.Y. and Roy Chowdhury, J. (1992) *J. Biol. Chem.* 267, 963-967.
6- Zhu, N., Liggit, D., Liu, Y. and Debs, R. (1993) *Science,* 261, 209- 211.
7- de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. and Appelmans, F. (1955) *Biochem. J.* 60, 604-617.
8- Beaufay, H., Jacques, P., Baudhuin, P., Sellinger, O.Z., Berthet, J. and de Duve, C. (1964) *Biochem. J.* 92, 184-205.
9- Jadot, M., Wattiaux-De Coninck, S. and Wattiaux, R. (1985) Eur. J. Biochem.151, 485-488.
10- Bowers, W. E., Finkenstaedt, J.T. and de Duve, C. (1967) *J. Cell Biol.* 32, 325-327.
11- Wattiaux, R., Wibo, M. and Baudhuin, P. (1963) *Ciba Found. Symp. "Lysosomes",* 176-200.