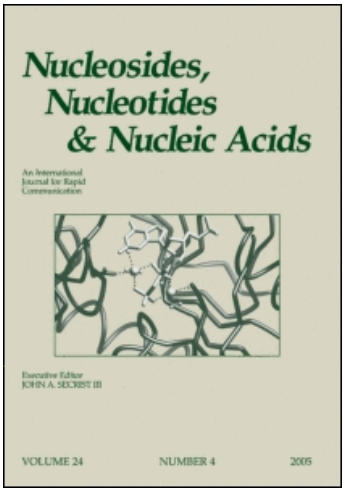

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III. PRECLINICAL STUDIES OF OLIGOMERS

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DELIVERY OF POLYNUCLEOTIDES WITH POLYAMINE LIPIDS AND POLYMERS

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Abstract: Several non-permanent polycations possessing substantial buffering capacity below physiological pH, such as lipopolyamines and polyethylenimines, are efficient transfection agents *per se*, i.e. without the addition of lysosomotropic bases, or cell targeting or membrane disruption agents. These vectors have been shown to deliver genes as well as oligonucleotides both *in vitro* and *in vivo*. Our hypothesis is that their efficiency relies on extensive endosome swelling and rupture that provides an escape mechanism for the polycation/DNA particles.

Introduction.

Gene transfer is the weak link of gene therapy and DNA is a pro-drug rather than the therapeutic effector molecule itself. The cascade of events leading to the synthesis of a large number of therapeutic protein molecules from a single gene begins in the nucleus. Therefore vector systems are required to carry the exogeneous DNA through the plasma and nuclear membranes. Most often, recombinant viral vectors are used for this task (1), since viruses have evolved sophisticated break-in ways (2) that can now be exploited. These include efficient cell membrane rupture mechanisms and nuclear targeting. Membrane rupture can occur either directly at the cell surface or after endocytosis. In any case the viral fusogenic protein becomes 'informed' of the cell proximity and undergoes a major conformational change induced either by binding to a cell surface receptor or by the acidic nature of the endosomal compartment. Chemists examining such complex molecular sequences may well be daunted. Yet synthetic non-viral gene transfer systems, however basic, will be of great potential to the gene therapy field just as soon as they show *sufficient* in vivo transfection capacities. We insist on the term *sufficient*, - the same adjective is

apparently used to describe the Rolls-Royce engine in the technical notice accompanying the car!- as it suggests an adequate performance. However, as emphasised by the recent British and American attempts to apply gene therapy to cystic fibrosis or melanoma patients, we know that this criterion is far from being satisfied (1b).

Chemistry is not constrained by the need for replication characteristic of a biological system and can therefore explore and exploit a much wider spectrum of candidate molecules for a given task. With some imaginative leads and a great deal of 'evolutionary' trial and error, two classes of synthetic vectors have been developed over the last decade. These compounds, whether lipids (3) or polymers (4), are all cationic like their classical predecessors used for in vitro transfection (calcium phosphate, DEAE-dextran). On complexing with DNA they cause several plasmid molecules to condense together into sub-micrometric particles. Oligonucleotides share many features with genes, being rather fragile polyanionic molecules with intracellular targets. Yet their much smaller size leads to smaller complexes and allows molecules to diffuse in the cytoplasm. Thus oligonucleotides delivery looks simpler and results achieved with genes, which is the main topic of this discussion, will *a fortiori* apply to oligonucleotides.

In vitro transfection with cationic lipids (5).

When a cationic lipid is used at an excess ratio of cationic charges to nucleic acid phosphates, the resulting nucleo-lipid particles will fix to the cell surface. Indeed, in vitro, electrostatic interactions between the positively charged DNA/lipid complexes and anionic heparan sulfate proteoglycans of the cell membranes are enhanced by increasing the overall charge of the complexes, which is in turn achieved by increasing the ratio of lipid to DNA. This interaction between the particle and the cell membrane is spontaneously followed by endocytosis. Cationic lipids give variable transfection efficiencies that depend both on the chemical structure of the vector and on the cell type. Even so, irrespective of the cell type, the lipopolyamines constitute one of most efficient vector classes (3, 6). This general efficiency is an intrisic property of the charged headgroup, and the addition of neither fusogenic lipids or of nuclear localisation signals can increase it, suggesting that the polyamine headgroup may in itself carry these multifunctional properties. Moreover, when the potentiometric protonation states of the amines were measured, it was found that at physiological pH only three of the four nitrogens in the spermine head were cationic (Figure 1). The pKa of the last amine is 5.5, half way between the extracellular and intralysosomial pH values, a clue to a possible buffering property that could well be exploited, and a point we shall return to later.

In vitro transfection with cationic polymers.

Compared to the lipopolyamines, most members of the other class of cationic molecules - the cationic polymers such as poly-L-lysine - are relatively inefficient unless conjugated to a ligand that can provoke endocytosis when bound to its cell surface located receptor (4). Even then transfection levels only reach those obtained with with lipopolyamines when the polymer is conjugated to adenoviral particles that lyse endosomes (9). However, the use of the non-specific adenovirus components

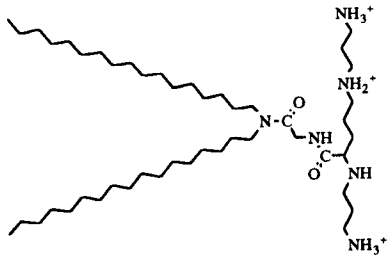

Figure 1. Chemical structure of the lipopolyamine Transfectam at neutral pH.

counteracts the cell targeting function obtained with the ligand. Recently, an exemplary class member of the cationic polymers has emerged with the description of the transfection properties of the polyamidoamine dendrimers (10). These quasispherical macromolecules bear a large number of amine groups on their surface and again, as for the lipopolyamines, not all of these amines are protonated at physiological pH.

So by observing two completely different cationic vectors, a lipid and a polymer, we are led to the same question: whether there is a causal relation between the overall buffering capacity of a vector under physiological conditions and its transfection possibilities. Accordingly, a number of macromolecular compounds bearing high amine group densities were considered for synthesis. Such cationic compounds would still be able to compact DNA, but owing to the repulsion predicted between like charges at close proximity, they would not be fully protonated at physiological pH.

As it turned out there was no need to start synthesizing candidates, as the ideal molecule was already available. In the commercially available polymer polyethyenimine (PEI, Figure 2) one in every three atoms is an amine group and the overall protonation level increases from 20 to 45% between pH 7 and 5. Moreover, the compound was described over 50 years ago and its innocuity demonstrated by its intensive and various uses: in water purification, ore extraction and in shampoos. We tested the transfection efficiency of this polymer, comparing it to lipopolyamines on a large variety of cell lines and primary cultures (12). The results are most promising, showing efficiencies at least as high as the best currently available synthetic vectors (Figure 3) indicating an entirely new function for this simple molecule.

Gene transfer in vivo.

Results obtained *in vivo* are quite encouraging too. The main limitation of current non-viral gene transfer methods is their low efficiencies *in vivo*. This is particularly marked for the cationic polymers like poly-L-lysine. As to the cationic lipids, such as Lipofectin, DOTAP, DC-Chol and Transfectam, which have been on the market for eight years or so, only recently have reports appeared showing their potential use for *in vivo* gene therapy situations (1b). Transfectam can provide

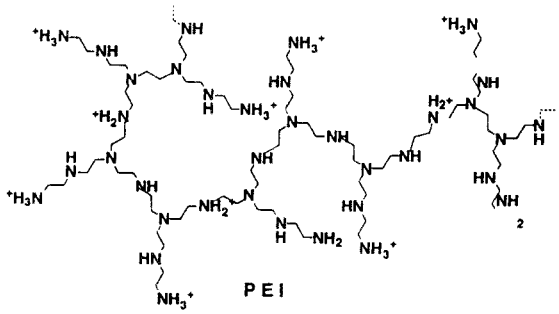

Figure 2. Chemical structure of polyethyleneimine at neutral pH .

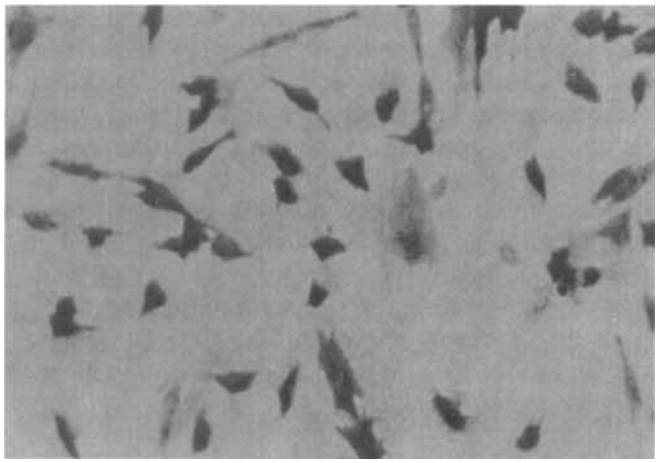

Figure 3. Expression of ß-galactosidase following transfection (2 µg CMV-βgal) with PEI in the murine 3T3 fibroblast cell line.

reasonable levels of transfection when used alone, without the adjonction of a neutral lipid, in the chick embryo (13) and in the mouse embryo transfected through introduction of DNA complexed with lipid into the maternal blood supply (14). In the newborn mouse brain we found (16) high levels of transfection using Transfectam at a low charge ratio with respect to the DNA and compensating the low overall amount of lipid (17) by the addition of a two-fold molar excess of the zwitterionic lipid, dioleoyl phosphatidylethanolamine (DOPE). Other authors have also reported that mixtures of cationic lipids and DOPE used at low overall charge ratios can deliver genes in vivo, either with Lipofectin (which is commercialised as a mixture of a cationic lipid and DOPE, see for example ref 15) or with Transfectam (18).

In the newborn mouse brain PEI/ DNA complexes have been shown to provide levels of transfection equal to those found *in vitro* for the same amount of DNA applied to primary neuronal cultures (11, Figure 4). Moreover, in more compact tissues such as

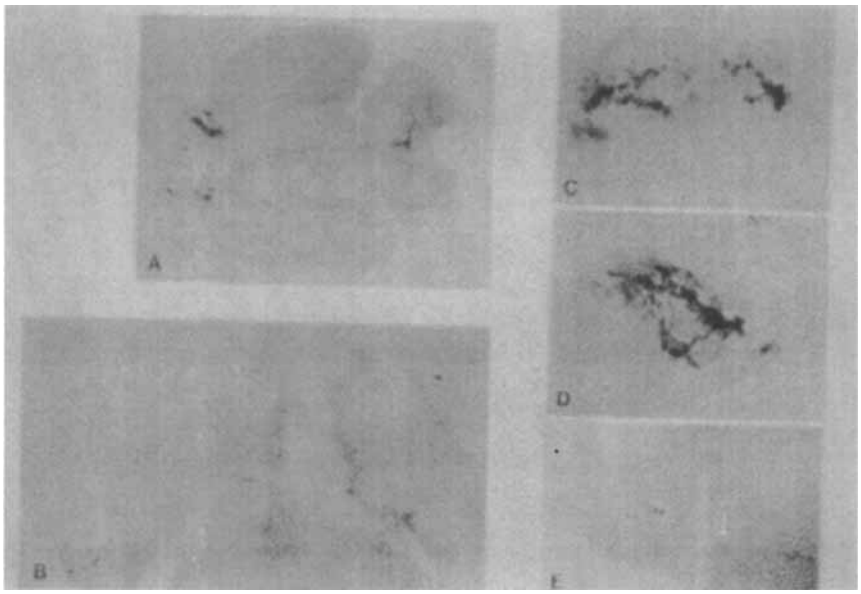

Figure 4. Spatial distribution of ß galactosidase expression in the newborn mouse brain after transfection of 1µg CMV-ßgal complexed with PEI (volume 2µl) into the striatum. A: low power overall view of the clarified brain, B: higher power view of the cerebral hemispheres, C and D: higher power views of the olfactory bulbs, E: higher power view of neurons labelled in the cerebral cortex. Photo: B. Abdallah, C. Benoist, MNHN, Paris.

the adult mammalian brain where cationic lipids provide either very low levels of transfection (16) or are actually worse than naked DNA, PEI also improves delivery. It can be complexed with DNA in various volumes (1 to 5µl) and used to transfect either delimited structures or larger regions of the adult mouse brain (19). Use of double immunostaining with antibodies against cell specific markers and transgene products show that both neurons and glia can be transduced by PEI transfection *in vivo*. Acute toxicity seems to be low, as no mortality nor necrosis at the site of injection has been observed in injected animals. A final point of interest is that when transfecting neuronal cells in culture with genes as well as with oligonucleotides, not only could gene expression and antisense effects be demonstrated, but also no interference with membrane excitability was seen (20). Thus, PEI appears to be an ideal vector for gene transfer into the brain *in vivo* and future studies will show if can be adapted to other tissues.

Gene transfer mechanism (5).

The sequence of events that we hypothesize to account for the remarkable transfection properties of PEI are summarized in Figure 5. The polycation/DNA

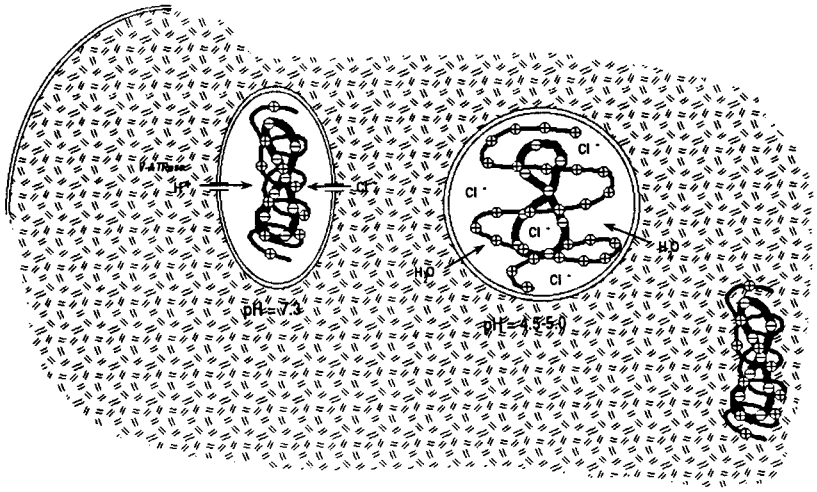

Figure 5. The proton sponge hypothesis: H^+ and Cl^- entry into the endosome leads to osmotic swelling and finally to endosome rupture.

complexes enter the cell by spontaneous endocytosis. A complex wholly covered in positive charges interacting with the cell membrane will produce a high local concentration of PEI in the endosome. During the intracellular trafficking the buffering capacity of the PEI will not only tend to inhibit the action of the lysosomal nucleases that have an acid optimal pH, but will also alter the osmolarity of the vesicle. The accumulation of protons brought in by the endosomal ATPase is coupled to an influx of chloride anions (21). In the presence of PEI there will be an large increase in the ionic concentration within the endosome resulting in osmotic swelling of the endosome. Moreover PEI protonation will also expand its polymeric network by internal charge repulsion (22). With the two phenomena occuring simultaneously it is likely that endosomal life expectancy is sorely reduced! Taking into account the protonation profile of PEI we can expect that about a third of the nitrogen atoms in the molecule participate in the swelling action, making the molecule a virtual proton sponge. For gene therapy the interesting aspect of this mechanism (which is somewhat 'primitive' compared to the mechanisms developed by viruses) is that it will lead to enhanced gene transfer, as the DNA introduced with the PEI will be rapidly liberated from the damaging endosomal environment. Thus, this molecule constitutes, per se, a promising vector for gene therapy and an ideal structural base for constructing more sophisticated vectors that could include supplementary functions such as cell-specific targeting ligands.

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