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Drug targeting: synthesis and endocytosis of oligonucleotide-neoglycoprotein conjugates

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ABSTRACT

Inhibition of gene expression by antisense oligonucleotides is limited by their low ability to enter cells. Knowing that sugar binding receptors, also called membrane lectins, efficiently internalize neoglycoproteins bearing the relevant sugar, 6-phosphomannose, for instance, oligonucleotides—substituted on their 5′-end with either a fluorescent probe or a radioactive label on the one hand, and bearing a thiol function on their 3′-end, on the other hand,—were coupled onto 6-phosphomannosylated proteins via a disulfide bridge. The oligonucleotide bound to 6-phosphomannosylated serum albumin is much more efficiently internalized roughly 20 times than the free oligonucleotide. Although most of the oligonucleotides are associated with vesicular compartments, oligonucleotides after releasing from the carrier by reduction of the disulfide bridge may find their way to reach the cytosol and then lead to an increase in the efficiency of the oligonucleotides.

INTRODUCTION

Antisense oligodeoxyribonucleotides have been reported to act in *in vitro* experiments as inhibitors, for virus or cellular gene expression by forming hybrids with mature mRNAs or pre-mRNAs (for reviews see: 1,2). The major limitation of a putative use of unmodified antisense oligonucleotides as therapeutic agents are their low ability to enter cells. Oligonuleotides have been shown to be taken up by cultured cells through a saturable and energy dependent process putativly mediated by a membrane receptor or transporter, but this process does not allow the concentration of the oligonucleotides inside the cells and therefore leads to low intracellular concentrations (3, 4).

Several modifications have been proposed to increase the intracellular concentration of oligonucleotides. When oligonucleotides are substituted with hydrophobic compounds such as acridine (5), cholesterol (6,7), alkyl chain (8,9) or lipid (10) or bound to a polycation such as poly(*L*-lysine) (11–13) their penetration into cells and or their biological activity are improved.

Cells expressed surface receptors mediating endocytosis of their ligands, receptor-specific macromolecular carriers are good candidates to target oligonucleotides to selected cells and to increase their accumulation in intracellular compartments. Amongst cell surface receptors, various sugar binding receptors (so called membrane lectins) have been characterized at the cell surface of a large number of normal and malignant cells (for reviews see: 14–17). These cell surface receptors specifically bind and internalize glycoproteins and neoglycoproteins bearing an appropriate sugar residue. For instance, hepatocytes express a galactose specific membrane lectin (18), macrophages a mannose specific membrane lectin (19,20) but also a mannose-6-phosphate specific membrane lectin (21). Monocytes which lack the mannose receptor express the mannose-6-phosphate specific membrane lectin (22); after few days in culture, monocyte derived macrophages bind and internalize with a great efficacy neoglycoproteins bearing either mannose or mannose-6-phosphate residues. Two mannose-6-phosphate receptors have been characterized: a 275 000 Mr receptor which bind M6P even in absence of cation is a membrane lectin involved both in lysosomal enzyme trafficking and in the endocytic pathway and a 46 000 Mr receptor which requires calcium or other divalent cations to bind M6P and glycoproteins bearing M6P (23), is a membrane lectin only involved in the intracellular trafficking.

Neoglycoproteins (serum albumin substituted with glycosides) and glycosylated neutral polymers (gluconoylated poly-*L*-lysine substituted with glycosides) which are specifically recognized and endocytosed by membrane lectins, have been used as drug carriers. Cytotoxic drugs and antiviral drugs have been targeted to malignant cells (24–26) or virus-infected hepatocytes (27 and references therein), but the main successfull targeting was achieved with macrophages and macrophage lineage cells. Macrophages can be activated and become tumoricidal both *in vitro* and *in vivo* by muramyldipeptide bound to neoglycoproteins

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or polymers bearing mannose residues at concentrations 100 to 1000-fold less than when free muramyldipeptide is used (28–31). Macrophages serve as a host for a large number of intracellular pathogens, parasites and viruses: recently it was shown that antiparasite and antiviral drugs bound to glycosylated carriers had a better activity that the related free drug (32,33). On these bases, such drug delivery system could selectively enhance oligonucleotide uptake by macrophages.

As macrophages act as a reservoir for HIV (for a review see 34), we decided to target antisense oligonucleotides by using glycosylated macromolecular carriers.

The oligonucleotide was substituted at both 3'- and 5'-ends in order to increase its stability and to allow its linkage to the neoglycoprotein on one hand and the addition of a fluorescent or radioactive probe on the other hand. In the oligonucleotide neoglycoprotein conjugates, the oligonucleotide 5'-end is substituted either with a fluoresceinyl residue or with a ^{125} I iodo-tyramine residue, the oligonucleotide 3'-end is linked through a disulfide bridge to the neoglycoprotein. The binding and internalization of oligonucleotideneoglycoprotein conjugates were assessed using peritoneal mouse macrophages as well as immortalized cell lines as models:—a murine macrophage cell line, clone J774 E which is a variant of the J774 cells obtained by Diment *et al* and choosen because this clone expresses the mannose specific lectin (35) as well as the cation independent M6P specific lectin (unpublished results),—U937, human promonocyte cell line and -BHK cells, fibroblast like cell line known to express the cation independent M6P specific membrane lectin (36).

In this paper, we describe the preparation of oligonucleotide-neoglycoprotein conjugates and we determine the cell association and the internalization of the oligonucleotides and the neoglycoprotein conjugates and we study the intracellular localization of these conjugates. For the reasons indicated above, the carrier is a protein substituted with mannose-6-phosphate residues, M6P-BSA which bind to and is internalized by cells expressing mannose-6-phosphate specific membrane lectins. The oligonucleotide is a 19 mer 2 deoxy-β-ribonucleotide, which hybridizes the [+64 +83] sequence of HIV-1 Bru LTR (37) and inhibits the expression of a reporter enzyme in cells transfected with constructs containing LTR linked to the cDNA of that enzyme (38). This 19 mer was selected as a model for further investigation on the biological activity of the oligonucleotide conjugates.

We show that the intracellular concentration of the 19mer oligonucleotide was much higher when the cells were incubated with the oligonucleotide bound to the 6-phosphomannoside-bearing neoglycoprotein than when the cells were incubated either with the free oligonucleotide or with the oligonucleotide bound to sugar-free albumin.

MATERIALS AND METHODS

Chemicals

1,1'-carbonyldiimidazole (CDI), *p*-formaldehyde and TLC silica gel 60 F_{254} aluminium sheets were purchased from Merck (Darmstadt, Germany); tyramine (Tya) and dithiotreitol (DTT) from Aldrich (Strasbourg, France); 1,4-diazabicyclo [2,2,2] octane (DABCO) from Sigma (Saint Louis, MO, USA); Fluorescein isothiocyanate (FITC isomer I) from Molecular Probes (La Jolla, CA, USA); ^{125}I Na from (Amersham, Buckinghamshire, UK), *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP), 4-succinimidyloxycarbonyl-α-methyl-α-(2-pyridinyldithio)-toluene (SMPT) and Iodogen from Pierce (Rockford, IL, USA). Enzymes, *Penicillium citrium* nuclease P1 and calf intestine alcaline phosphatase were obtained from Boehringer (Mannhein, Germany). Biogel P-2 was purchased from Bio-Rad (Richmond, CA, USA); Trisacryl GF05 from Sepracor (Villeuneuve-la Garenne, France) and G-75 from Pharmacia (Uppsala, Sweden). The neoglycoprotein M6P-BSA was prepared by coupling 16 *N*-[*p*-(*O*-6-phospho-α-D-mannopyranosyl)phenyl]thiocarbamyl groups to BSA according to Roche *et al* (22). All solvents were dried and distilled before use.

Oligodeoxyribonucleotides were synthesized on a Milligen DNA synthesizer 75000 (Milligen, St Quentin en Yvelines, France) using the phosphoramidite method (39). Ion exchange HPLC were carried on Mono P 5/5 column with a FPLC GP 250/500 apparatus (Pharmacia). Oligonucleotides were analyzed by reverse phase HPLC on a Lichrocart column (125 mm×4 mm) packed with 5μm Lichrospher and purified on a Lichrocart column (250 mm×25) mm packed with 7μm Lichrospher 100 RP-18 (Merck) using a Waters G 25 LC system apparatus and a Waters 660 E system controller equipped with a Waters 990 photodiode array detector (St Quentin en Yveline, France). Gel electrophoresis were carried out on a LKB 2010 Macrophor apparatus equipped with a 2297 macrodrive 5 generator (Pharmacia) or on a BRL S2 appartus (Bethesda Research Laboratories, Bethesda, USA) equipped with an EPCS 3000/150 (Pharmacia).

Synthesis of the 5'-fluorescein, 3'-disulfide bridge-substituted 19 mer oligonucleotide (5'F-19mer-RSSR-OH3', 4. R= -(CH2)2-O-(CH2)2-O-(CH2)2. Assembly of the nonadecamer d(5'AAGCTTTATTGAGGCTTAA3') complementary to the sequence [+64;+83] of the $LTR_{HIV\text{-}Bru}$ was performed on a disulfide derivatized solid support (10 μmol scale) (Fig 1) (40). Detritylated 5'-end of the otherwise fully protected oligomer **1** (uncleaved from the support) was activated for 5 h at 20°C with 1.5 mmol 1,1'-carbonyldiimidazole (CDI) in 5 ml dioxane to afford compound **2** which then reacted for 12 h with 2.75 mmol hexamethylene diamine in 5 ml pyridine (41). After cleavage from the support and deprotection with concentrated ammonia for 12 h at 50°C, the oligonucleotide **3**, 5' aminohexylamino carbonyl, 3'disulfide bridge nonadecamer, NH_2-$(CH_2)_6$-NH-CO-d(5'AAGCTTTATTGAGGCTTAA3')-$(CH_2)_2$-O-$(CH_2)_2$O-$(CH_2)_2$-S-S-(CH_2)-$_2$-O-$(CH_2)_2$-O-$(CH_2)_2$-OH was purified by ion exchange chromatography on a Mono P 5/5 column equilibrated with a mixture of 0.01 M phosphate buffer, pH 6.8 and of CH_3CN (4:1; vol/vol). The product was eluted with a linear gradient from 0 to 1.5 M NaCl in 20 min (Rt_3= 14.8 min, flow rate 1μl/min). CH_3CN was evaporated under reduced pressure and compound **3** was desalted on Biogel P_2column eluted with ditilled water and freeze-dried. Fluorescein isothiocyanate (FITC, 10 μmol in 200 μl DMF) reacted for 5 h at 20°C with the amino group linked to the 5'-end of **3** (0.5 μmol) in 2 ml of 0.1 M carbonate/bicarbonate buffer, pH 9.5. Then, 0.7 ml 4M ammonium acetate was added and the oligonucleotide derivative was purified by precipitation from its aqueous solution by adding 10 volumes of ethanol in order to remove the excess of free fluorescein isothiocyanate remaining in the supernatant. The pure compound **4** (F-19mer-RSSR-OH) with R= -$(CH_2)_2$-O-$(CH_2)_2$-O-$(CH_2)_2$- was obtained after reverse phase

chromatography on Lichrocart (12.5 cm×4 cm) packed with 5 μm Lichrospher RP18 (Merck) using a linear gradient of CH_3CN (8.75 to 35%) in 0.1 M aqueous triethylammonium acetate (TEAA) buffer, pH 7.0 (Rt_4= 12.7 min, flow rate 4 ml/min, in 30 min).

*Synthesis of 5'-iodo-tyramine, 3'disulfide bridge 19 mer oligonucleotides. *Tya-19mer-RSSR-OH, 7.* Tyramine (3.5 mmol in 5 ml dry pyridine) reacted for 12 h at 20°C with the nonadecamer **2** activated on its 5'-end. The excess of tyramine was removed by several washing of the resin with pyridine and the oligonucleotide was cleaved from the support and deprotected by treatment for 12 h at 50°C with concentrated ammonia as described above. The compound **6** (Tya-19mer-RSSR-OH) with R= $-(CH_2)_2-O-(CH_2)_2-O-(CH_2)_2-$ was precipitated by adding 10 volumes ethanol and purified by reverse phase chromatography on Lichrocart (12.5cm×4cm) packed with 5m m Lichrospher RP18 (Merck) using a linear gradient of CH_3CN (5 to 27.5%) in 0.1 M TEAA buffer, pH 7.0 (Rt_6= 17.8 min, flow rate 4 μl/min), desalted on Biogel P_2 column eluted with distilled water and finally freeze-dried. The compound **6** was iodinated using iodogen as oxidative reagent (42). ^{125}I Na (37 MBq) was added to 0.15 μmol oligonucleotide in 100 μl PBS and the mixture was stirred for 10 min at 20°C. The reaction was stopped by addition of 100 ml tyrosine 1 mM in PBS. After 10 min, the radiolabelled oligomer **7** (*Tya-19mer-RSSR-OH, 440 MBq/μmol) was purified by gel filtration on a Biogel P2 column equilibrated with PBS. The same procedure was used to introduce a radioiodinated tyramine group on a 16mer oligonucleotide d($^{5'}T_4CT_4C_6T^{3'}$) synthesized from 5'-dimethoxytrityl-thymidine-3'-CPG (Merck).

Enzymatic degradation of the iodinated oligonucleotide **6** was performed in order to check the specificity of the radiolabelling on the tyramine residue as previously described (43). Briefly, a solution of 20 μg of *Penicillium citrium* nuclease P1 in 60 μl 6.6mM $ZnSO_4$, 0.18 M sodium acetate buffer, pH 5.3 was mixed with *Tya-19mer-RSSR-OH **7** (0.6 MBq) in 10 mwater and kept for 1 h at 20°C. Then, the solution pH was raised up to 8 by adding 1 M Tris and 20 μl calf intestine alcaline phosphatase was added. The solution was kept at 37°C for 2h, then heated at 100°C for 5 min. Released products were analyzed by thin layer chromatography, using isopropanol/ammoniac/water (7:1:2, per volume) as solvent.

Preparation of BSA and M6P-BSA bearing dithiopyridinyl residues. $M6P_{16}$-, PDP_{15}-BSA and PDP_{10}-BSA were prepared as previously described (44) by using SPDP (45). In order to prepare conjugates with stable disulfide bridges, $M6P_{16}$-BSA and BSA were substituted with eight 4-carbonyl-α-methyl-α-(2-pyridinyldithio)-toluene (MPT) residues by using 4-succinimidyloxycarbonylα-methyl-a (2-pyridinyldithio)-toluene (SMPT) (46), leading to $M6P_{16}$-, MPT_8BSA and MPT_8-BSA, respectively. The number of sugar residues bound per BSA molecule was determined by the resorcinol/sulfuric acid method (47) and the number of PDP or MPT molecules bound per BSA or M6P-BSA molecules were determined from absorbance at 343 nm in the presence of DTT (45).

Preparation of oligonucleotide-neoglycoprotein conjugates. *Tya-19mer-RSSR-OH **7** or F-19mer-RSSR-OH **4** oligonucleotides (0.8 mg; 0.12 mmol) in 200 μl of 0.1 M NaCl, 1.7 M EDTA, 0.1 M sodium acetate buffer, pH 4.5 were treated with DTT (3 mg; 19 μmol) for 6 h at 20°C under nitrogen to generate a 3'-thiol function. DTT and HS-R-OH were then removed by five extraction with 2 ml ethyl acetate (saturated with H_2O and outgassed by bubbling nitrogen). The radiolabelled oligonucleotide **8** (*Tya-19mer-RSH) was added dropwise to 2mg (0.025 mmol) $M6P_{16}$-, PDP_{15}-BSA or PDP_{10}-BSA in 0.5 ml, 0.1 M NaCl, 1.7 M EDTA, 0.1 M sodium phosphate buffer, pH 7.2. Similarly, the fluoresceinylated oligomer **5** (F-19mer-RSH, 0.8 mg; 0.12 mmol) was added dropwise to $M6P_{16}$-, MPT_8-BSA or MPT_8-BSA (2 mg; 0.025 μmol). The reaction was conducted for 6 h at 20°C under nitrogen. Conjugates were purified by gel filtration on Sephadex G75 column (1 cm×25 cm) in PBS.

The purity of each conjugate was checked by gel electrophoresis on 20% PAGE Fastgel using 'native' buffers strips (Pharmacia). The gels were fixed for 30 min in 20% acetic acid, 5% glycerol solution, and dried in air. Fluorescent compounds were revealed by silver staining while radiolabelled products were revealed by autoradiography for 10 h at −80°C using Fuji X-ray films.

Protein concentration was determined according to the Bradford method (48). The number of radioactive oligonucleotide molecules per conjugate molecule was calculated by measuring the radioactivity of the oligonucleotide-neoglycoprotein conjugate.

The number of fluorescenylated oligonucleotide molecules per conjugate molecule was determined by measuring the fluorescence intensity at 520 nm upon excitation at 495 nm of a solution of oligonucleotideneoglycoprotein conjugate pretreated with 0.1 M DTT for 2h. The fluorescence intensity of 1 μg/ml fluoresceinylated oligonucxleotide was used as standard.

Oligonucleotides and conjugates stability in culture media. Radiolabelled oligomer **7** (*Tya-19mer-RSSR-OH) free and bound to M6P-BSA ($M6P_{16}$-, [*Tya19mer]$_{1.3}$-BSA) were incubated at 37°C in 200 μl of MEM medium supplemented with 5% of heat inactivated fetal bovine serum. Aliquots (10 μl) were collected every 2 h and immediately frozen at −20°C. Samples were concentrated by using a speed-vac concentrator (Savant instruments, N.Y., USA) and analyzed by 20% PAGE electrophoresis in the presence of 7 M urea (running conditions: 2000 V for 30 min and 2 h at 1500 V). Gels were dried and revealed by autoradiography at −80°C for 10 h.

Cells

Peritoneal macrophages. (C57BL/6×Balb/c) F_1 mice (6−7 weeks old) purchased from CSEAL (CNRS,Orléans, France) were injected with 2 ml thioglycolate medium (Institut Pasteur, Paris, France). Four days later, peritoneal macrophages were harvested by three lavages of the peritoneal cavity with 5 ml RPMI 1640. Cells were spun down and resuspended in 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ PBS containing 1% BSA at pH 7.4 (complete PBS).

Cultured cells. Human promonocytes (U937 cells), kindly provided by Dr. L. Gazzolo (Lyon, France), were grown in suspension in RPMI 1640 medium. The murine macrophage cell line J774 clone E (J774E) cells kindly provided by Dr. P. Stahl (Saint Louis, MO, USA) (35) and baby hamster kidney cells, BHK cells were cultured in monolayers in RPMI 1640 and in MEM media, respectively. The media were supplemented with 2 mM L-glutamine, 100 units/μl penicillin, 0.1 μg/ml streptomycin and 10% of heat inactivated fetal bovine serum.

Cells were grown at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air and were *Mycoplasma* free as evidenced by using BVC-kanamycin A staining method (49). Adherent cells were harvested during their exponential growing phase by treatment with 2.5 μg/ml trypsin and 0.02% (w/v) EDTA in PBS.

Cellular uptake of free or conjugated radiolabelled oligonucleotides. Cells (2×10^5/well) were incubated either at 4°C or at 37°C in a microtitration plate previously coated with BSA (2% in PBS for 1 h at 37°C) with 200 μl PBS containing different concentrations of radiolabelled oligonucleotide either free or bound to 6-phosphomannosylated BSA. After 2 h, the cells were harvested by gentle pipetting, put onto 150 μl dibutylphtalate/bis(2-ethylhexyl)phthalate (3:1; v/v) mixture in a 500 μl propylene tube and centrifuged for 5 min at 10 000 g (Beckman Microfuge, Gagny, France) in order to remove unbound ligands (50). The tube tip, containing the cell pellet, was cut and the cell associated radioactivity was counted in a LKB 1282 Compugamma counter (Pharmacia).

Flow cytometry analysis of binding and endocytosis of free and conjugated fluoresceinylated oligonucleotides. Plated J774E cells or peritoneal murine macrophages (2×10^5/well) were incubated at 37°C in 200 μl RPMI containing fluoresceinylated oligonucleotide free or bound to either BSA or M6P-BSA. After incubation, cells were washed with PBS and suspended in sheath fluid (51). The cell fluorescence intensity was measured using a FACS Analyzer (Becton Dickinson, Sunnyvale, CA, USA)

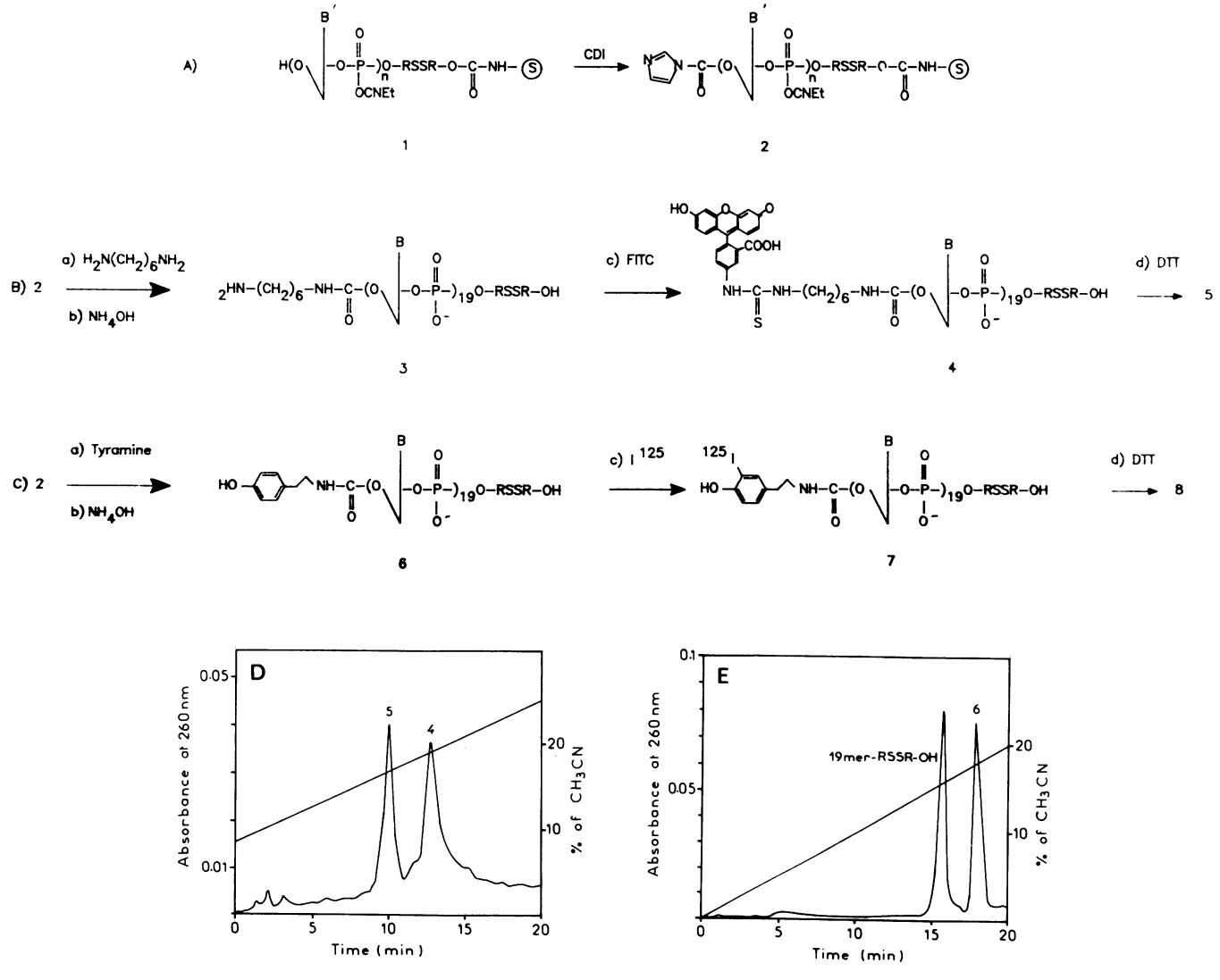

Figure 1. Oligonucleotide synthesis. A: The 5'OH group of the 19mer oligonucleotide **1** still otherwise fully protected and bound to the modified solid support is activated by reaction with CDI to give the compound **2**; B: Synthesis of the 5'fluoresceinylated 19 mer oligonucleotide bearing a 3'-thiol function **5**. (a) compound **2** was treated with hexamethylene diamine and then (b) deprotected with NH_4OH. (c) oligonucleotide **3** reacted with FITC to afford the 5' fluoresceinylated oligomer **4** bearing a 3'-disulfide arm. (d) The masked 3'-thiol group of **4** was liberated by reduction with DTT; C: Synthesis of the 5'-radioiodinated 19mer oligonucleotide bearing a 3'thiol function **8**.(a) compound **2** was reacted with tyramine and then (b) deprotected by treatment with NH_4OH. (c) oligonucleotide **6** substituted at the 5'-end by a tyramine group **6** was radiolabelled by ^{125}I. (d) The masked 3'-thiol function of **7** was liberated by treament with DTT; D, E: Reverse phase HPLC chromatogram on Licrosorb RP-18 (5μm) column (3×150 mm); flow rate 1ml/min; D: Compounds **4**, **5** eluted with a linear gradient of CH_3CN (8.75 to 35%) in 0.1M aqueous TEAA buffer pH 7 (Rt_4= 12.7 min; Rt_5= 10.1 min). E: **6** and 5'-non substituted 19mer (19mer-RSSR-OH) eluted with a linear gradient of CH_3CN (5 to 27.5%) in 0.1M TEAA buffer pH 7 ($Rt_{19mer\text{-}RSSR\text{-}OH}$= 15.7 min; Rt_6= 17.8 min). B= nucleic base; B'= protected nucleic base; CDI= 1,1'-carbonyldiimidazole; CNEt= cyanoethyl; DTT= dithiotreitol; FITC= fluorescein isothiocyanate; R= $-(CH_2)_2-O-(CH_2)_2-O-(CH_2)_2-$; -NH-(S) = aminopropylFractosyl 500.

equipped with a FACSLite unit (Becton Dickinson): excitation wavelength at 488 nm from a 25 mW air cooled argon laser; emitted light collected through a 520 ± 10 nm interference filter. The following three parameters 90° light scatter, electric volume and green fluorescence, were simultaneously recorded in list mode on a minimum of 5000 cells at 300 events per second. The data were analyzed in biparameter cytograms using the Consort 30 device (Hewlett Packard). The cell fluorescence intensity was measured before and after a post-incubation at 4°C for 30 min in the presence of 50 μM monensin (Calbiochem, La Jolla, CA, USA) (25,51). The posttreatment at 4°C in the presence of H^+/Na^+ ionophore neutralizes the acidic compartments, reversing the acid dependent quenching of fluorescein fluorescence; the comparison of the fluorescence intensity of cells post incubated in the presence and in the abscence of monensin gives informations about the intracellular position of the endocytosed material. The cell fluorescence intensity values were expressed as a mean value statistically calculated from the fluorescence intensity of each cell presents in a gated population. The cell fluorescence intensity was expressed by relatively to that of calibrated beads (25) allowing a quantitive estimation of the cell associated fluorescence and to make valuable the comparison of the cell fluorescence obtained in the separate experiments.

Confocal microscopy. J774E cells were plated on glasses (2×10^5/slide) and cultured for further 24 hr. Then the culture medium was discarded, cells were washed with complete PBS containing 1% BSA and incubated for 3 h at 37°C with 2.5 μM fluoresceinylated 19mer oligonucleotide either free or bound to BSA or to M6P-BSA. Cells were washed with PBS to remove unbound ligands, fixed with 1% *p*-formaldehyde in PBS for 1 h at room temperature and mounted in a PBS/glycerol mixture (1:1; v/v) containing 1% of DABCO (Sigma) as an anti fading agent (52). Cells were analysed with a confocal fluorescence microscope (Lasersharp MRC-600, Bio-Rad, Oxfordshire, England) at 488 nm from an Argon laser.

RESULTS

In order to increase the cellular uptake of oligonucleotides, oligonucleotides were coupled to neoglycoproteins specifically endocytosed *via* membrane lectins of macrophages. Mannose-6-phosphate was chosen instead of mannose in order to target macrophages as well as their precursors (monocytes, U937 cells) which are known to have no mannose receptor on their surface but to express one mannose-6phosphate specific lectin. To follow the cellular uptake, conjugates bearing either fluorescent or radioiodinated oligonucleotides were prepared.

*Oligonucleotide synthesis (F-19mer-RSSR-OH **4**; *Tya-19mer-RSSR-OH **7**).* An oligonucleotide d(5'AAGCTTTATTGAGGCTTAA3') complementary to the [+64,+83] sequence of $LTR_{HIV\text{-}BRU}$ and bearing a disulfide bridge on its 3' end was synthesized by solid phase synthesis using a previously described solid support (40). This oligonucleotide was modified on the 5'-end in order to introduce either a fluoresceinyl or a phenolic residue. The 5'-OH group of the oligonucleotide still attached on the solid support **1** was activated with CDI (**2**) (fig 1A).

*F-19mer-RSSR-OH **4**.* FITC was coupled on an aminohexamethylene arm introduced at the 5'-end of the oligonucleotide *via* a non-biodegradable carbamate linkage as shown in figure 1B.

Tya-19mer-RSSR-OH **7.* A tyramine group, which can be easily iodinated was introduced at the 5'-end of the oligonucleotide using a two step procedure (see fig 1C). After complete deprotection, compound **6** (Tya-19mer-RSSROH) was easily separated from 19mer-RSSR-OH oligonucleotide by reverse phase HPLC (fig 1E) and was obtained in a 80% yield. Compound **6** was radiolabelled by using ^{125}I Na together with iodogen. Upon enzymatic degradations of *Tya-19mer-RSSR-OH with *Penicillium citrium* nuclease P1 and then with calf intestine alcaline phosphatase a single radioactive spot (Rf= 0.79) close to d(A) (Rf= 0.74) was detected by TLC. The migrations of d(C), d(G) and d(T) were slower ($Rf_{d(C)}$= 0.59, $Rf_{d(G)}$= 0.56, $Rf_{d(T)}$= 0.67). As the tyramine residue is borne by a d(A) in the *Tya19mer-RSSR-OH, this result suggests that the oligomer was selectively radioiodinated on the tyramine group.

*Oligonucleotide-protein conjugates M6P-, [*Tya-19mer]-BSA, *Tya-19mer-BSA conjugates and M6P-, [F19mer]-BSA, [F-19-mer]-BSA conjugates.* Oligonucleotides with a thiol group at their 3' end were coupled to BSA and M6P-BSA bearing pyridyldithiopropionyl groups introduced by using either SPDP or SMPT: The 3'-thiol function of F-19mer-RSSR-OH **4** and of *Tya-19mer-RSSR-OH **7** was produced by reduction of the disulfide bridge with DTT. The reaction was monitored by reverse phase HPLC. As shown in fig 1D, the F-19mer-RSH **5** was eluted before its precursor F-19mer-RSSR-OH **4**. The 3'-thiol function was reacted with BSA or M6P-BSA bearing dithiopyridinyl groups, leading to conjugates with 1 or 2*Tya oligonucleotides and about 1 F-oligonucleotide per BSA or M6P-BSA molecule.

The purity of each conjugate and the nature of the linkage between oligonucleotide and protein were checked by 20% polyacrylamide gel electrophoresis before and after reduction with DTT(fig 2A and 2B). The radiolabelled compound corresponding to *Tya-19mer bound to M6P-BSA (fig 2A, lane 1) upon DTT treatment migrated (fig 2B, lane 1) with the rate of *Tya-RSH (fig 2B, lane 3) showing that the radiolabelled oligonucleotide was initially linked to M6P-BSA by a disulfide bridge sensitive to DTT.

Oligonucleotides and conjugates stability in culture medium

The stability of oligonucleotide bound to M6P-BSA in culture medium was monitored by PAGE. M6P-, [*Tya-19mer]-BSA was incubated at 37°C in MEM medium supplemented with 10% heat inactivated fetal bovine serum. Aliquots were collected upon incubation for 2 or 4h and analyzed by electrophoresis (fig 3). In the presence of heat inactivated fetal bovine serum, the radioactivity did not leave the radiolabelled oligonucleotide-

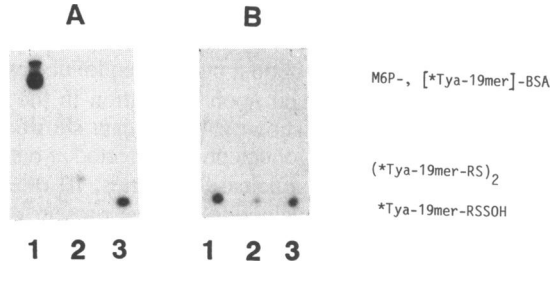

Figure 2. Autoradiograms of $M6P\text{-}_{16}$-,[*Tya-19mer]-BSA (1), (*Tya-19mer-$RS)_2$ (2) and *Tya-19mer-RSSR-OH (3) after electrophoresis on 20% polyacrylamide gel before (A) and after (B) a DTT treatment.

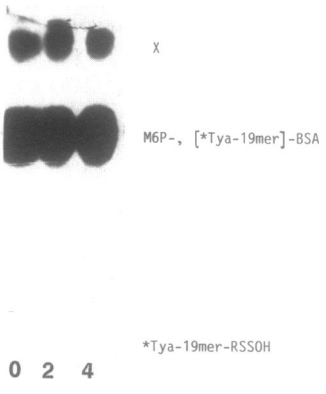

Figure 3. Stabilities of oligonucleotide-conjugates in culture medium. M6P[*Tya-19mer]-BSA was incubated at 37°C in MEM medium supplemented with 10% heat inactivated fetal bovine serum. Aliquots were collected immediatly (t=0) or after 2h (t=2) or 4h (t=4) incubation at 37°C and were analysed by electrophoresis in 20% polyacrylamide gels in presence of 7M urea. Gels were dried and autoradiographied. X: stands for adsorbed material of very high molecular mass.

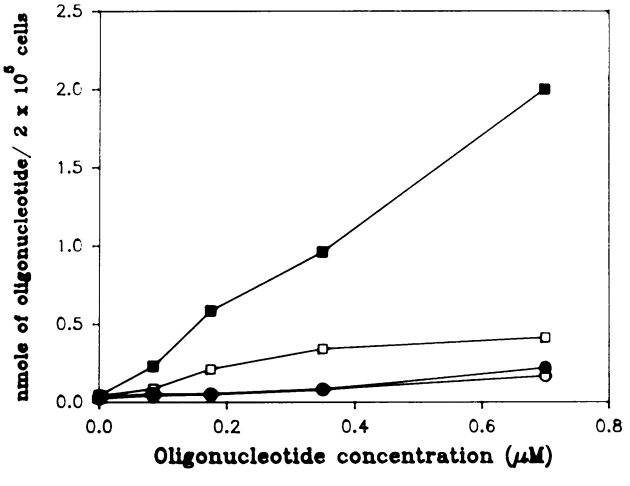

Figure 4. Amount of radioiodinated oligonucleotide associated with mouse peritoneal macrophages after incubation with 19mer oligonucleotide either free or bound to M6P-BSA. Macrophages were incubated for 2 h at either 4°C (□, ○) or at 37°C (■, ●) in the presence of *Tya19merRSSR-OH (○, ●) or $M6P_{16}$-,[*Tya-19mer]-BSA (□, ■). The cell associated radioactivy was counted after removing unbound ligands by centrifugation through an oil cushion (see Materials and Methods).

neoglycoprotein conjugates (fig 3) showing that the oligonucleotide was neither released as such from the conjugate nor released as small parts upon a putative endonuclease action. Similar results were obtained upon incubation in the presence of mouse serum (data not shown). These data showed that, in a culture medium, i) an oligonucleotide protected at both 5′- and 3′-ends is prevented from nuclease activities, ii) the disulfide bridge between the oligonucleotide and the protein is stable.

Cell association of radiolabelled 19mer oligonucleotide free or bound to M6P-BSA

The amount of cell-associated oligonucleotide was assessed upon incubation at 4°C with the 19mer oligonucleotide free or bound to M6P-BSA. M6P-BSA specifically bind M6P specific

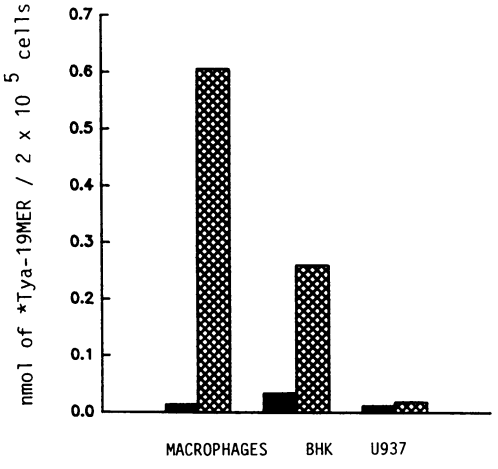

Figure 5. Amount of *Tya-19mer oligonucleotide either free or bound to M6P-BSA associated with macrophages, BHK or U937 cells. Cells were incubated for 2 h either at 4°C or 37°C with 0.35 μM free *Tya-19mer (■) or 0.35 μM M6P-BSAbound *Tya-19mer oligonucleotide (⊠). Unbound ligands were removed by microcentrifugation through an oil cushion (see Materials and Methods). The intracellular concentration of oligonucleotide was calculated by substracting the radioactivity associated to cells upon incubation at 4°C from the radioactivity of cells upon incubation at 37°C.

membrane lectins present on the cell surface of the selected target cells. The amount of the radiolabelled 19mer oligonucleotide bound at the cell surface of murine peritoneal macrophages upon incubation at 4°C was lightly higher when cells were incubated with the 19mer oligonucleotide-neoglycoprotein conjugate than with the free 19mer oligonucleotide (fig 4): the plateau was reached when cell were incubated with 0.35 μM conjugate. Upon incubation at 37°C with the free 19mer oligonucleotide to allow endocytosis, the cell-associated oligonucleotide did not significantly increase, whereas incubation at 37°C with M6P-, *Tya-19mer-BSA a large amount of oligonucleotide was associated with macrophages (fig 4).

Experiments were conducted with other cells expressing M6P membrane lectins, such as the promonocytic cell line U937 and the fibroblast-like BHK cells (fig 5). Although U937 are known to express M6P receptors on their surface (53), most of the M6P receptors are located on the intracellular membranes and consequently these cells poorly internalized M6P bearing proteins, and the uptake of 19 mer oligonucleotide coupled to M6P-BSA was only 1.5 higher than that of free 19mer oligonucleotide. After substracting the amount of oligonucleotide bound to U937 cells upon incubation at 4°C, the intracellular concentration of 19mer oligonucleotide free or bound to M6P-BSA was estimated, assuming a cell volume of 1.76×10^{-6} μl (15 μm diameter) to be about 0.032 μM and 0.055 μM, respectively (fig 5). Therefore, the oligonucleotide concentration was not significantly different. In contrast, BHK cells as well as murine peritoneal macrophages accumulated a higher amount of 19mer oligonucleotide when 19mer oligonucleotide was bound to M6PBSA. The oligonucleotide intracellular concentration in BHK cells and in macrophages was 0.73 μM and 1.7 μM, respectively, higher than the oligonucleotide concentration in the incubation medium (0.35 μM). Conversely, with cells incubated with the free 19mer oligonucleotide the intracellular concentration was very low, 0.095 μM in BHK cells (one fourth of the oligonucleotide concentration in the incubation medium) and 0.03 μM in

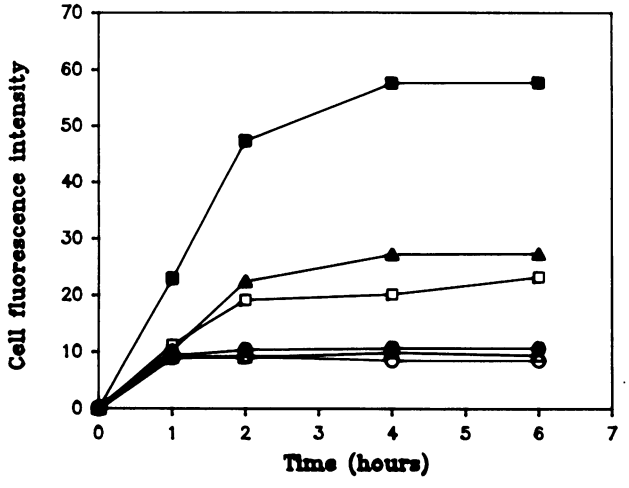

Figure 6. Uptake of 5'-fluoresceinylated 19mer oligonucleotide-neoglycoprotein conjugates by J774E cells. J774E cells were incubated at 37°C in the presence of either 0.35 μM F-19mer (○, ●), 0.35 μM F-19mer bound to BSA (△, ▲, i.e. 60μg/ml) or bound to M6P-BSA (□, ■, i.e. 70μg/ml). The cell fluorescence intensities were measured by flow cytometry before (○, △, □) and after a post-incubation for 30 min at 4°C with 50 μM monensin (●, ▲, ■).

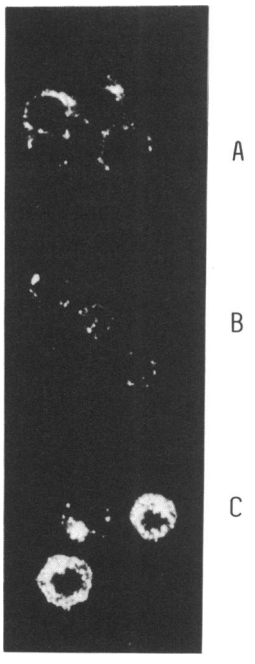

Figure 7. Localization inside J774E cells of fluoresceinylated 19mer oligonucleotide-neoglycoprotein conjugates. Plated cells were incubated for 3 h at 37°C with 2.5 μM of F-19mer oligonucleotide free (A) or 1 μM F-19mer oligonucleotide bound to BSA (B, i.e. 200μg/ml conjugate) or 0.5 μM F-19mer oligonucleotide bound to M6P-BSA (C, i.e. 100μg/ml conjugate). Then, cells were washed with PBS, fixed with 1% *p*-formaldehyde, mounted in PBS/glycerol mixture containing 1% DABCO and finally observed with a confocal microscope. Pictures represented are a central section of the cells.

macrophages (one tenth of the oligonucleotide concentration in the incubation medium). The oligonucleotide intracellular concentration of BHK cells and macrophages incubated with 19mer oligonucleotide bound to M6P-BSA is, respectively, 8 and 50 times higher than the intracellular concentration of cells incubated the free 19mer oligonucleotide.

Uptake of fluoresceinylated conjugates by J774E cells and murine peritoneal macrophages

The uptake of fluoresceinylated oligonucleotides free or bound to either BSA or M6P-BSA was assessed by flow cytometry using a method previously described (25, 51). J774E cells were incubated at 37°C with 0.35 μM fluoresceinylated oligonucleotides either free or bound to the neoglycoprotein, the cell fluorescence intensity was measured at different times over a 6 h period, before and after monensin postincubation (fig 6). Oligonucleotide internalization upon incubation at 37°C was demonstrated by the increase of cell associated fluorescence intensity followed by the post-incubation at 4°C in the presence of monensin: because fluorescein fluorescence is quenched in acidic environment, and because the endocytosed material is rapidly associated with acidic vesicles (endosomes and/or lysosomes), monensin is used to raise the intravesicular pH and therefore to overcome the fluorescein fluorescence quenching. The fluorescence of cells incubated with F-19mer oligonucleotide remained low even after 6 h incubation and the fluorescence did not increase after monensin post treatment at 4°C indicating that the oligonucleotide was not or very poorly internalized by an endocytosis process. The cell associated fluorescence upon incubation with oligonucleotide bound to BSA was roughly as the level of that obtained with cells incubated in the presence of free F-19mer oligonucleotide, however the cell associated fluorescence increased after addition of monensin suggesting that the conjugate was internalized into acidic compartments. The uptake of oligonucleotide bound to BSA or to M6P-BSA, measured after monensin post incubation reached a plateau after 4 h of incubation. The fluorecence of J774E cells incubated with the oligonucleotide bound to Man6PBSA was higher than the fluorescence of J774E cells incubated with oligonucleotide bound to BSA before and after monensin post-incubation. Oligonucleotide concentration after 2hr incubation at 37°C was found to be 0.06 μM, 0.68 μM and 1.43 μM for free oligonucleotide and oligonucleotide bound to BSA and M6PBSA, respectively. This significant increase of oligonucleotide uptake is in agreement with an endocytosis process mediated by mannose-6-phosphate specific membrane lectins of J774E cells.

Similar results were obtained with murine peritoneal macrophages incubated for 2h with oligonucleotide bound to BSA or Man6P-BSA: the fluorescence increased after monensin post incubation and the cellassociated fluorescence was 7 and 12 times higher when macrophages were incubated with 1 μM F19mer oligonucleotide bound to BSA and M6PBSA respectively than when macrophages were incubated with 1 μM free 19mer oligonucleotide (data not shown).

Confocal microscopy

The intracellular localization of both free and conjugated oligonucleotide was determined using a fluorescence confocal microscope (fig 7). J774E cells incubated for 3 h at 37°C with 2.5 μM F-19mer oligonucleotide were lightly labelled, fluorescence was mainly located on the plasma membrane and very few vesicles were observed. The fluorescence of cells incubated with 1 μM F-19mer linked to BSA was similar and located close to the cell periphery , whereas cells incubated with 0.5 μM F-19mer oligonucleotide conjugated to M6P-BSA were strongly fluorescent and the fluorescence was mainly located in vesicules.

DISCUSSION

A 19mer oligodeoxyribonucleotide was synthesized and modified on both the 3′ and 5′-ends. The oligonucleotide was substituted at the 3′-end by a thiol group in order to couple it onto a protein carrier bearing activated thiol groups. The 5′-end was either fluoresceinylated or radioiodinated to monitor oligonucleotide internalization into the cells. A new simple method was used to radioiodinate the oligonucleotide. Generally, $TiCl_4$ or iodogen are used to radioiodinate cytosine of DNA or RNA (54,55). Knowing that carbone-iode bond in a benzene ring is more stable than in heterocycles, *p*-methoxyphenyl groups (56) or tyramine (57), coupled to a cytosine residue, have been introduced into oligonucleotides as iodinable aromatic groups. In this paper a tyramine was introduced at the 5′-end of the oligonucleotide *via* a carbamate linkage, at the last step of the synthesis on solid phase. The oligodeoxyribonucleotide 5′-end still linked on the support was selectively detritylated, the free 5′-hydroxyl group was activated with CDI and tyramine was added. 5′-Tyramine derivatized oligonucleotides were obtained with a 75% yield and the method is suitable to prepare such derivative by using an automatic DNA synthesizer.

The 5′-radioiodinated or 5′-fluoresceinylated oligodeoxyribonucleotides were conjugated to 6-phosphomannosylated BSA *via* a disulfide bridge using either SPDP (44) or SMPT to obtain more stable disulfide linkages (46). In average, 1 or 2 oligonucleotide molecules were bound onto BSA and M6P-BSA bearing PDP or MPT groups. This type of linkage has been largely used in drug targeting to prepare immunotoxins and was shown to be cleaved inside the cells. Shen *et al* (58) reported that MTX linked to undegradable poly(***D***-lysine) *via* a disulfide bridge was as toxic as MTX bound onto poly(***L***-lysine) *via* an amide linkage, showing an intracellular reduction of the disulfide. Recently, the same authors showed that cleavage of the disulfide began near the cell surface during the first step of endocytosis , and occur also later in the Golgi apparatus (59). A plant toxin, gelonin, a strong inhibitor of protein synthesis in a cell-free system, but a mild inhibitor of cells was found to be 100 fold more toxic as a gelonin-neoglycoprotein conjugate because of selective endocytosis *via* membrane lectins of Lewis lung carcinoma cells (24).

Unmodified oligonucleotides are usually completely degraded within 15 min in calf serum (60), on the contrary oligonucleotides modified at both 3′ and 5′-ends are stable in culture medium. In culture media, the oligonucleotide stability depends mainly on 3′-exonuclease and to a lesser extend to the 5′ exonuclease activity (1). We show here that the conjugate, in which the 5′-radioiodinated oligodeoxyribonucleotide is bound onto a protein (BSA or M6P-BSA) *via* a disulfide linkage is stable in the culture medium supplemented with 10% heat inactivated fetal bovine serum i.e, the disulfide bridge is not reduced and the oligonucleotide remains intact. Oligonucleotides (0.35 μM) are slowly taken up by cells, independently of the cell type (macrophages, J774E, BHK, or U937 cells), the uptake of the 19mer oligonucleotide remained very low even upon 24 h incubation. The internalization of this oligonucleotide coupled to M6P-BSA was higher than the uptake of free oligonucleotide and depended upon the cell type: macrophages internalized twice more conjugates than BHK and 30 fold more than U937 cells. The cellular uptake of oligonucleotide M6PBSA conjugate was mediated by mannose-6-phosphate membrane lectins present at the surface of each cell type. These results are in agreement with the endocytosis capacity of these cells which is high with macrophages, moderate with BHK and low with U937 cells as demonstrated by using fluoresceinylated neoglycoproteins. When cells are incubated with oligonucleotide at micromolar concentration, the oligonucleotide concentration inside the cells is about 10% of the concentration in incubation medium (61,4). Accordingly, when macrophages or the murine macrophage cell line, J774E, are incubated for 2 h with 0.35 μM of fluoresceinylated or radiolabeled 19mer oligonucleotide, the intracellular concentration of the oligomer was very low (0.04−0.06 μM). Under the same conditions, the 19mer bound onto M6P-BSA was internalized through their membrane lectins, leading to a high intracellular concentration (1.43 to 1.7 μM), i.e the intracellular concentration was 4- to 5-fold higher than the extracellular concentration. Therefore the use of Man6P-BSA as a cell specific carrier in cells expressing the M6P receptor on their surface improve the uptake about 30-fold. The high level of uptake is in the range of that obtained with oligonucleotide substituted with compounds such as cholesterol, phospholipids or poly(***L***-lysine) which are not cell specific. Attachment of a cholesterol residue at the 3′-end of a decathymidylate increased the oligonucleotide internalization by a factor of 6 to 8 when 2.5 μM and 0.5 μM cholesterol derivatized oligonucleotide were incubated for 2 h with L929 or Krebs-2 cells, respectively (7). Attachment of a phospholipid to the 5′-end of a 15mer oligonucleotide increased 10 fold its cellular uptake after a 4 h incubation (10). Attachment of a fluoresceinylated 15 mer oligonucleotide to poly(***L***-lysine) increased 12 fold its internalization by L929 cells (12). Recently, the uptake by brain capillaries of a biotinylated 21mer antisense oligonucleotide was increased 4 fold when the biotinylated oligomer was complexed with avidin (62). In this last case, it is the cationic property of avidin which mediates the uptake as in the case of polylysine. The above modifications increased the oligonucleotide cellular uptake, but did not provide a selective targeting; conversely glycoconjugates which are recognized by specific membrane lectins are expected to selectively target specific cells. The cellular uptake of antisense oligonucleotides linked to glycosylated macromolecules was highly increased in comparison with the uptake of free oligonucleotides. Studies using fluorescence confocal microscopy showed that the fluoresceinylated oligonucleotide was mainly associated with vesicular compartments as expected from an endocytotic process. However as it is difficult to quantitate the dull fluorescence found in the cytosol, we are now assessing the amount of oligonucleotide present in the cytosol and/or in the nucleus of cells previously incubated with radiolabelled oligonucleotides and conjugates, by subcellular fractionations. The uptake of phosphodiester oligodeoxyribonucleotides which has been reported as to be mediated by cell surface molecules acting as oligonucleotide receptors (3), or the uptake of methylphosphonate oligonucleotides which is due to fluid phase endocytosis or even to non-specific adsorbtive endocytosis (63) lead to a distribution of oligonucleotides confined into vesicular compartments. As biological effects have been reported with antisense oligonucleotides directed against viral sequences, it is expected that a small amount of the oligonucleotides which have been taken up into vesicles has reached its target mRNA in the cytosol after crossing an intracellular membrane. It could be expected that increasing the intracellular concentration may also result in increasing the amount of oligonucleotide able to cross a membrane to reach the cytosol. The 19mer oligonucleotide with

a sequence complementary to a part of the LTR_{HIV}, and the oligonucleotide-neoglycoprotein conjugates are being tested on macrophage like cells transfected with LTR_{HIV} governing the luciferase gene, as reporter gene.

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ABBREVIATIONS

BSA: bovine serum albumin; CDI: 1,1′ carbonyldiimidazole; DABCO: 1,4 diazabicyclo(2,2,2, octane); M6PBSA: 6-phospho-mannosyl bovine serum albumin; DTT, dithiothreitol; HIV: human immunodeficiency virus; LTR: long terminal repeat; PBS: phosphate buffered saline, pH 7.4. SMPT: 4-succinimidyloxy-carbonyl-amethyl-(2-pyridinyldithio)-toluene; SPDP= *N*-succinimidyl 3-(2-pyridinyldithio)-propionate; *Tya-19mer= oligonucleotide $^{5'}$AAGCTTTATTGAGGCTTAA$^{3'}$ substituted at the 5′-end with a ^{125}I,iodinated tyramine residue (*Tya) and at the 3′-end with a thiol group. TEAA:triethylammonium acetate.

REFERENCES

1. Uhlman E. and Peyman A. (1990) Chem. Rev. **90**, 543–584.
2. Helene C. and Toulme J.J. (1990) Biochim.Biophys.Acta, **1049**,99–125.
3. Loke S.L., Stein C.A., Zhang X.H., Mori K., Nakanishi M., Subasinghe C., Cohen J.S. and Nekers L.M. (1989) Proc. Natl. Acad. Sci. USA **86**, 3474–3478.
4. Yakubov L.A., Deeva E.A., Zarytova V.F., Ivanova E.M., Ryte A.S., Yurchenko L.V. and Vlassov V.V. (1989). Proc. Natl. Acad. Sci. USA **86**, 6454–6458.
5. Verspieren P., Cornelissen A.W.C.A., Thuong N.T., Helene C. and Toulme J.J. (1987) Gene **61**, 307–315.
6. Letsinger R.L., Guangrobg Z., Sun D.K., Ikeuchi T. and Sarin P.S. (1989) Proc. Natl. Acad. Sci. USA **86**, 6553–6556.
7. Boutorin A.S., Gus'kova L.V., Ivanova E.M., Kobetz N.D., Zarytova V.F., Ryte A.S., Yurchenko L.V. and Vlassov V.V. (1989) FEBS Letters **254**, 129–132.
8. Kabanov A.V., Vinogradov S.V., Ovcharenko A.V., Krivonos A.V., Melik-Nubarov N.S., Kiselev V.I. and Severin E.S. (1990) FEBS Letters **259**, 327–330.
9. Saison-Behmoaras T., Tocque B., Rey I., Chassignol M., Thuong N.T. and Helene C. (1991) EMBO J. **10**, 1111–1118.
10. Shea R.G., Marsters J.C. and Bischofberger N. (1990) Nucl. Acids Res. **18**, 3777–3783.
11. Lemaitre M., Bayard B. and Lebleu B. (1987) Proc. Natl. Acad. Sci. USA **84**, 648–652.
12. Leonetti J.P., Degols G. and Lebleu B. (1990) Bioconjugate Chem. **1**, 149–153.
13. Stevenson M. and Iversen P. (1989) J. Gen. Virol. **70**, 2673–2682.
14. Monsigny M., Kieda C. and Roche A.C. (1979) Membrane lectins. Biol.Cellulaire **36**, 289–300.
15. Monsigny M., Kieda C. and Roche A.C. (1983) Biol. Cell **47**, 95–110.
16. Monsigny M., Roche A.C., Kieda C., Midoux P. and Obrenovitch A. (1988) Biochimie **70**, 16331649.
17. Sharon N. and Lis H. (1989) Science **246**, 227–233.
18. Ashwell G. and Harford J. (1982) Annu. Rev. Biochem. **51**, 531–554.
19. Stahl P.D., Rodman J.S., Miller M.J. and Schlesinger P.H. (1978) Proc. Natl. Acad. Sci. USA **75**, 1399–1403.
20. Shepherd V.L. and Sthal P.D. (1981) Proc. Natl. Acad. Sci. **78**, 1019–1022.
21. Shepherd V.L., Lee Y.C., Schlesinger P.H. and Stahl P.D. (1984) J. Biol. Chem. **259**, 2257–2261.
22. Roche A.C., Midoux P., Bouchard P. and Monsigny M. (1985) FEBS Letters **193**, 63–68.
23. Kornfeld S. (1987) FASEB. J. **18**, 462–468.
24. Roche A.C., Barzilay M., Midoux P., Junqua S., Sharon N. and Monsigny M. (1983) J. Cell Biochem. **22**, 131–140.
25. Monsigny M., Roche A.C. and Midoux P. (1984) Biol. Cell. **51**, 187–196.
26. Duncan R., Kopeckova-Rejmanova P., Strohalm J., Hume I., Cable H.C., Phol J., Lloyd J.B. and Kopecek J. (1987) Br. J. Cancer **55**, 165–174.
27. Fiume L., Busi C., Mattioli A. and Spinosa G. (1988) CRC Crit. Rev. Ther. Drug Carriers Syst. **4**, 265283.
28. Monsigny M., Roche A.C. and Bailly P. (1984) Biochem. Biophys. Res. Commun. **121**, 579–584.
29. Roche A.C., Bailly P. and Monsigny M. (1985) Invasion metastasis **5**, 218–232.
30. Derrien D., Midoux P., Pepit C., Negre E., Mayer R., Monsigny M. and Roche A.C. (1989) Glycoconjugate J. **6**, 241–255.
31. Petit C., Monsigny M. and Roche A.C. (1990) J. Biol. Response Modifiers **9**, 33–43.
32. Chakraborty P., Bhaduri A.N. and Das P.K. (1990) Biochem. Biophys. Res. Commun. **166**, 404–410.
33. Midoux P., Negre E., Roche A.C., Mayer R., Monsigny M., Balzarini J., De Clerq E., Mayer E., Ghaffar A. and Gangemi J.D. (1990) Biochem. Biophys. Res. Commun. **167**, 1044–1049.
34. Meltzer M.S., Skillman D.R., Gomatos P.J., Kalter D.C. and Gendelman H.E. (1990) Annu. Rev. Immunol. **8**, 169–194.
35. Diment S., Leech M.S. and Stahl P.D. (1987) J. Leucocyte Biol. **42**, 485–490.
36. Gabel C.A., Goldberg D.E. and Kornfeld S. (1983) Proc.Natl.Acad.Sci. USA **80**, 775–779.
37. Muesing M.A., Smith D.H. and Capon D.J. (1987) Cell. **48**, 691–701.
38. Boidot-Forget M. (1988) Thesis, Paris.
39. Beaucage S.L. and Caruthers M.H. (1981) Tetrahedron Lett. **22**, 1859–1862.
40. Bonfils E. and Thuong N.T. (1991) Tetrahedron. Lett. **32**, 3053–3056.
41. Watcher L., Jablonski J. and Ramachandran K.L. (1986) Nucl. Acids Res. **14**, 7985–7994.
42. Fraker P.J. and Speck J.C. (1978) Biochem. Biophys. Res. Commun. **80**, 849–857.
43. Asseline U., Thuong N.T. and Helene C. (1986) Nucleot. Nucleos. **5**, 45–63.
44. Breton P., Midoux P., Petit C., Bousser M.T., Roche A.C., Mayer R. and Monsigny M. (1991) Bioconjugate Chem. **2**, 16–18.
45. Carlsson J., Drevin H. and Axen R. (1978) Biochem. J. **173**, 723–737.
46. Thorpe P.E., Wallace P.M., Knowles P.P., Relf M.G., Brown A.N.F., Watson G.J., Knyba R.E., Warwrzynczak E.J. and Blakey D.C. (1987) Cancer Res. **47**, 5924–5931.
47. Monsigny M., Petit C. and Roche A.C. (1988) Anal. Biochem. **175**, 525–530.
48. Bradford M.M. (1976) Anal. Biochem. **72**, 248–254.
49. Monsigny M., Midoux P., Depierreux C., Befear C., Le Bris M.T. and Valeur B. (1990) Biol. Cell **70**, 101–105.
50. Connolly D.T., Townsend R.R., Kawaguchi K., Bell W.R. and Lee Y.C (1982) J. Biol. Chem. **257**, 939–945.
51. Midoux P., Roche A.C. and Monsigny M. (1987) Cytometry **8**, 327–334.
52. Johnson G.D., Davidson R.S., McNamee K.C., Russel G., Goodwin D. and Holborow E.J. (1982) J. Immunol. Methods **55**, 231–242.
53. Stein M., Zijderhand-Bleekemolen J., Geuze H., Hasilik A. and Von Figura K. (1987) EMBO J. **6**, 2677–2681.
54. Commerford S.L. (1971) Biochemistry **10**, 1994–2000.
55. Piatyszek M.A., Jarmolowski A. and Augustyniak J. (1988) Anal. Biochem. **172**, 356–359.
56. Dewanjee M.K., Ghafouripour A.K., Werner R.K., Serafini A.N. and Sfakianakis G.N. (1991) Bioconjugate Chem. **2**, 195–200.
57. Sauvaigo S., Fouque B., Roget A., Livache T., Bazin H., Chypre C. and Teoule R. (1990) Nucl. Acids Res. **18**, 3175–3183.
58. Shen W.C., Ryser H.J.P. and Lamanna L. (1985) J. Biol. Chem. **260**, 10905–10908.
59. Feener E.P., Shen W.C. and Ryser H.J.P. (1990) J. Biol. Chem. **265**, 18780–18785.
60. Wickstrom E. (1986) J. Biochem. Biophys. Methods **13**, 97–102.
61. Zamecnik P.C., Goodchild J., Taguchi Y. and Sarin P.S. (1986) Proc. Natl. Acad. Sci. USA **83**, 4143–4146.
62. Partridge W.M. and Boado R.J. (1991) FEBS letters **288**, 30–32.
63. Shoji Y., Aktar S., Periasamy A., Herman B. and Juliano R.L. (1991) Nucleic Acids Res. **19**, 5543–5550.