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Oligonucleotide delivery: Uptake of rat transferrin receptor antibody (OX-26) conjugates into an in vitro immortalised cell line model of the blood–brain barrier

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Abstract

Delivery of oligonucleotides (ODNs) to the brain is hindered by the tight junctions of the capillary endothelial cells that constitute the blood–brain barrier. We have examined the ability of a monoclonal antibody (OX-26), which recognises the rat transferrin receptor, to function as an effective carrier for the delivery of ODNs to the brain. In this initial report, we have characterised the uptake of OX-26–ODN conjugates in an immortalised, rat brain endothelial cell line, called RBE4, which is reported to be a good in vitro model of the blood–brain barrier. Uptake of the conjugate into this cell model was twofold higher than the free ODN and its uptake mechanism was consistent with transferrin receptor-mediated endocytosis. Exocytosis profiles of the OX-26–ODN conjugates were different from either free ODN or a non-specific IgG–ODN conjugate indicating an altered sub-cellular distribution, possibly involving 'deeper' cellular compartments. Treatment of cells with monensin further increased the intracellular accumulation of the OX-26–ODN conjugates suggesting that trafficking of the conjugate may involve the trans-Golgi network. These data suggest that OX-26 conjugates improve delivery of ODNs into an immortalised cell culture model of the BBB and are worthy of further study as carrier systems for the CNS delivery of nucleic acids.

Keywords: Oligonucleotide; Antisense; Delivery; Cell uptake; Transferrin receptor; Blood–brain barrier

1. Introduction

Antisense oligonucleotides (ODNs) are short, synthetic single strands of DNA that can inhibit gene expression by binding to complementary (and accessible) regions within a targeted mRNA

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sequence. These molecules may have potential applications as molecular biology tools to study gene expression and function as well as being used as novel therapeutic agents. Indeed, a number of clinical trials with ODNs are currently under way to examine their therapeutic potential in diseases such as AIDS and cancer (Szymkowski, 1996; Akhtar and Agrawal, 1997).

We have been investigating the potential application of antisense ODNs as anti-brain cancer agents and for the treatment of HIV infection within the central nervous system (CNS). Although antisense ODNs clearly exhibit pharmacological effects in the brain upon direct intracerebral injection (Suzuki et al., 1994; Wahlestedt, 1994), these large, and often polar, molecules do not readily enter the CNS following systemic administration (for a recent review, see Akhtar and Agrawal, 1997). This is due to their poor natural transport across the blood–brain barrier (BBB) which poses a formidable obstacle to the delivery of such non-lipophilic compounds (Goldstein and Betz, 1986; Pardridge, 1986). The cells of microvessel endothelium, which help form this barrier, are joined together by tight intercellular junctions that, together with the close proximity of the astrocytic cell processes, form a continuous wall against the passive movement of substances from the blood to the brain. The BBB ensures that the homeostasis of the CNS is maintained. However, the isolation of the brain from the bloodstream is not complete and specific transport systems within the capillary endothelial cells, such as those for amino acids, transferrin (iron), glucose and insulin, assures that the brain receives all the compounds required for its normal growth and function. The transferrin receptor, which efficiently transports iron into the brain, is highly expressed in the brain capillary endothelium (Jefferies et al., 1984). Therefore, antibodies directed against the transferrin receptor are highly specific reagents for targeting the brain capillary endothelium. Several studies have now shown that these antibodies are good carrier systems for drug delivery to the brain. The antibody OX-26 has previously been used in the CNS targeting of peptides (Friden et al., 1991; Bickel et al., 1993), polyamide nucleic acids (Pardridge et al., 1995) and ODNs via streptavidin/biotin interactions (Boado, 1995).

In this study, we have examined the covalently coupled antibody OX-26–ODN conjugates to function as a carrier for the delivery of antisense ODNs to brain endothelial cells by initially examining their uptake into an in vitro model of the BBB.

Cell culture models of brain endothelium are attractive candidates as in vitro BBB model systems since they have many fewer problems associated with binding and metabolism than encountered in in situ studies. However, it is difficult to grow primary brain endothelial cells free of any non-endothelial contaminant. The other major problem encountered in primary culture is the difficulty of obtaining good tight monolayers in a reproducible manner. For these reasons, the possibility of using immortalised cell lines has been investigated as an alternative. Rat brain microvascular endothelial cells prepared by the group of Françoise Roux have been successfully immortalised by Pierre-Olivier Couraud (Roux et al., 1993). One clone, called RBE4, has been further characterised. These cells displayed a nontransformed phenotype and expressed typical endothelial markers, Factor VIII-related antigen and *Bandeiraea simplicifolia* binding sites (Roux et al., 1993). When RBE4 cells were grown in presence of bFGF and on collagen-coated dishes, confluent cultures developed sprouts that extended above the monolayer and organised into capillary-like structures. The activity of the blood–brain barrier-associated enzyme, gamma-glutamyl transpeptidase (γ-GTP) and microvessel-related enzyme alkaline phosphatase (AP) could be detected in these tubular structures. RBE4 cells have been successfully grown on porous filter inserts (Romero et al., 1994), under conditions where astrocytes can be grown on the base of the culture wells below the filters. The permeability of the RBE4 monolayer to [^{14}C]sucrose was reported to be still greater than that of the BBB in situ but they approached the values reported for primary cultures of brain endothelial cells which have been used for studies of transendothelial permeability (Abbott et al., 1995). The absence of non-endothelial cell contaminants and the simplicity of

this immortalised cell line, preserving many important properties of the brain endothelium, constitute a good basis for an in vitro BBB model to study the uptake of antisense ODNs into the brain. In this initial report, we have characterised the binding and uptake of ODN conjugates into these cells in culture.

2. Methods

2.1. Synthesis and internal labelling of ODNs

ODNs were synthesised on an automated DNA synthesiser (Model 392, Applied Biosystems, Warrington, UK) using standard phosphoramidite chemistry (0.2 μM scale). A 5′-end C_6 thiol-modified 20-mer phosphodiester ODN antisense to the 3′-splice site of the tat gene in HIV-RNA (3′-NH_2-ACA CCC AAT T*CT GAA AAT GG 5′) that contained a single ^{32}P-labelled phosphodiester linkage at position 10 (shown as an asterisk) and an exonuclease resistant amine (NH_2-) group at its 3′-terminus was prepared as two fragments and subsequently ligated. The 3′-end 10 base fragment containing the amine-protecting group was synthesised using the 3′-Amine ON CPG column (Crauchem, UK) as this modification has been previously shown to block 3′-exonuclease activity (Zendegui et al., 1992). This fragment was then 5′-end labelled with [^{32}P]gamma-ATP (Amersham, UK) using T4 polynucleotide kinase (Bioline, UK) and purified by native 20% polyacrylamide gel electrophoresis. The 5′-end 10 base fragment was synthesised with and without a 5′-end trityl protected thiol group using the C_6-Thiol Modifier (Cruachem, UK). The two fragments were annealed to a 24-mer template (5′-TAT GTG GGT TAA GAC TTT TAC CAA 3′), a complementary ODN bearing two extra bases (underlined) at both ends making its molecular weight distinct from the antisense 20-mer. The 5′-end ^{32}P-labelled fragment was ligated to the other 10-mer using T4 DNA ligase (Bioline, UK) as described by Boado and Pardridge (1994). The resulting [^{32}P]20-mer was purified from the free template and unligated 10-mers by 7 M urea/20% polyacrylamide gel electrophoresis and passive elution. The purified 20-mer was then desalted on a NAP-10 column (Pharmacia Biotech, Sweden) equilibrated in water.

2.2. Preparation of antibody and derivatization with SMCC

OX-26 antibodies were purified from supernatants harvested from cultures of the OX-26 hybridoma cell line [purchased from the European Cell Culture Collection] by passage over a protein A-Sepharose CL-4B column (Pharmacia Biotech, Sweden) followed by elution under acid conditions and immediate neutralisation according to the manufacturer's protocol. The control human IgG antibody was purchased from Sigma. A 60 molar equivalent of the heterobifunctional cross linker, succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate was added to a solution of 750 μg IgG or OX-26 antibody in 0.5 ml of 50 mM phosphate buffer pH 8. The reaction mixture was incubated for 90 min at room temperature in the dark as described previously by Kuijpers et al. (1993). Excess SMCC was removed by gel filtration on Sephadex G-25 (3 cm × 1 cm) in 50 mM phosphate buffer pH 7 containing 0.1 M NaCl and 5 mM EDTA. The resulting concentration of the antibody was determined by measuring the absorbance at 280 nm (Kuijpers et al., 1993).

2.3. Conjugation of maleimide-antibody to thiol modified radiolabelled ODNs

The maleimide-antibody was conjugated to 10 molar equivalent of cold thiol modified ODN mixed with internally labelled thiol modified ODN according to the method of Kuijpers et al. (1993). The 5′-end thiol protecting group was previously removed with silver nitrate/dithiothreitol to generate a free thiol group according to the manufacturer's protocol (Cruachem, UK). Excess DTT was extracted with H_2O saturated ethyl acetate. The aqueous phase was immediately added to the purified fraction of maleimide derivatized antibody which had been thoroughly degassed under nitrogen. Conjugation was allowed to proceed overnight at room temperature.

Unreacted maleimide groups were blocked by the addition of 0.01 M cysteamine. Following another 2-h incubation at room temperature, the conjugate was purified by gel filtration on Sephacryl S-100 HR column (1.6 cm × 26 cm) using PBS as eluent. The first 1–2 ml fraction contained the purified conjugate as verified by 10% SDS-PAGE analysis. The quantity of free ODN in the eluate fractions was determined by measuring the absorbance at 260 nm. The number of ODN molecules conjugated to the antibody was deduced by subtracting the measured quantity of free ODN from the initial quantity of ODN used for the coupling.

2.4. Cell culture

RBE4 cells were provided by Dr P.O. Couraud (Institut Cochin, Paris). This immortalized cell line of rat brain microvascular endothelial cells preserves many features of the brain endothelium (Durieu-Trautman et al., 1993; Roux et al., 1993; Abbott et al., 1995). RBE4 cells were passaged twice a week in Alpha Medium/Ham's F10 (1:1, Life Technologies, UK) supplemented with 2 mM glutamine, 10% heat-inactivated foetal calf serum, and 1 ng/ml basic fibroblast growth factor (Boehringer Mannheim, Germany). They were spread at a density of 10^4 cells/cm^2, onto collagen-coated dishes (rat tail collagen type I, Sigma, UK) and used between passages 30 and 60. Cells were grown at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. The expression of the transferrin receptor on the cell surface of this cell line was established by histochemical staining. The primary human transferrin receptor antibody clone RVS-10 (Biogenesis, UK) was detected with Vectastain Elite ABC Kit (Vector, CA) as described in the manufacturer's protocol.

2.5. Cell uptake and efflux studies

For uptake experiments, 2×10^5 cells/well (24-well plate) were incubated with either internally labelled ODN-antibody conjugates or radiolabelled ODNs alone in serum-free cell culture medium. When competitors or monensin were used, these were added 15 min prior to addition of labelled antibody-ODN conjugates and during the incubation. After the desired incubation times (30 min to 5 h), the medium was removed and cells washed three times with 0.5 ml of 0.05% (w/v) PBS-azide. Cells were then lysed with 0.5 ml 3% triton X-100 and cell-associated radioactivity was determined by liquid scintillation counting using the same procedure as below. Uptake studies were performed either at 4°C or 37°C.

For efflux studies, the incubation medium was removed, cells washed three times with 0.5 ml PBS and fresh PBS was added. The release of free or conjugated radiolabelled ODN from cells was monitored at fixed intervals over 4 h. At each timed interval, 0.5 ml of PBS was removed and placed in 10 ml Optiphase Hi-Safe 3 (Pharmacia, UK) before being counted for 5 min in a Packard 1900 TR scintillation counter. An equivalent volume of fresh PBS was replaced at each sampling time.

3. Results and discussion

3.1. Conjugate synthesis

Antibody-ODN conjugates were prepared for both a control human IgG and a monoclonal antibody (OX-26) which recognises the rat transferrin receptor. In each case, the antibody was functionalized using the maleimide coupling strategy (Kuijpers et al., 1993; Walker et al., 1995) in which the heterobifunctional cross linker, succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC), enabled the introduction of reactive maleimide groups onto accessible amino groups. The internally ^{32}P-labelled ODN bearing a 3′-exonuclease resistant amine group and a protected thiol group at the 5′-end was prepared using the ligation strategy described in Methods (Section 2). Upon deprotection of the 5′-end thiol function by reduction with DTT, it was reacted with the maleimide groups of the antibody leading to the formation of radiolabelled ODN-antibody conjugates via a stable thioether linkage. The covalent coupling of the antibody–ODN conjugates was confirmed by electrophoretic mobility shift assays (EMSA) and by enzymatic characteri-

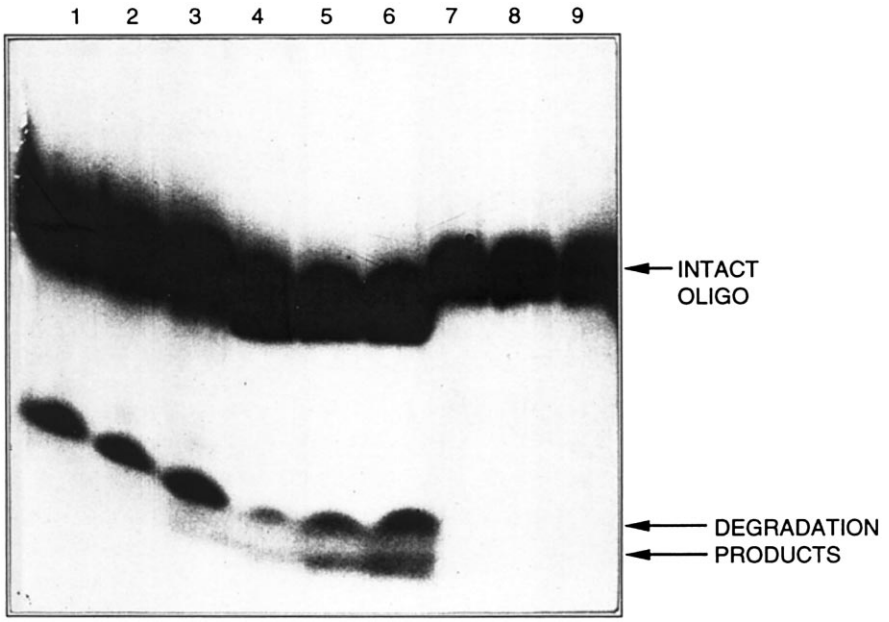

Fig. 1. Stability of 3′-end amine protected and unprotected ODNs when incubated with RBE4 cells in serum-free culture medium. Radiolabelled oligonucleotides (~ 100 nM) in serum-free medium were incubated with 1×10^4 RBE4 cells cm^{-2} on collagen-coated dishes as described in Methods (Section 2). At timed intervals, aliquots of the apical medium were removed and radiolabelled ODNs analysed by 20% PAGE and autoradiography. The stability profile of internally ^{32}P-radiolabelled 20-mer ODN (Lanes 7–9) is compared with two unprotected ODNs, a 5′-end ^{32}P-labelled 20 mer of identical sequence (Lanes 1–3) and a 17-mer sequence bearing a 3′-end ^{32}P-label (Lanes 4–6). Incubation times for Lanes 1 and 4 were 10 min, Lanes 2 and 5 were 20 min, Lanes 3 and 6 were 30 min, Lane 7 was 10 min, lane 8 was 1 h and lane 9 was 3 h.

sation as described by Walker et al. (1995) (data not shown). In our experience, typically 3–4 ODN molecules could be conjugated per antibody molecule which is a slightly lower ratio than reported by us previously for an antibody targeting the human transferrin receptor (Walker et al., 1995).

3.2. Stability of 3′-end protected ODN and time course of ODN–conjugate uptake

To ensure that the inclusion of a terminal 3′-amine group on the ODN was providing enhanced nuclease resistance, we compared the stability of 3′-end protected and unprotected oligonucleotides when exposed to RBE4-cells grown in serum-free culture media as this was the incubation protocol used in subsequent uptake studies (see below). Fig. 1 shows that 3′-end protected ODN was stable throughout the period of study whereas with unprotected ODN degradation products were visible within 10 min exposure to cells. These data support the findings of Zendegui et al. (1992) who showed that the presence of a 3′-amine function provides significantly enhanced resistance to 3′-exonuclease digestion.

Having confirmed its nuclease resistance, internally ^{32}P-labelled ODN either free (in 3′-amine protected form) or covalently-linked to the control IgG or the anti-rat transferrin receptor antibody (OX-26) was incubated at the final concentration of 1 μM for up to 5 h with RBE4 cells at 37°C. Cell uptake was assessed by measuring the cell associated radioactivity as determined by liquid scintillation counting. The percentage of associated radioactivity increased significantly during the 5 h of incubation (Fig. 2). The cellular association of the radiolabelled OX-26–ODN conjugate was up to twofold higher than that those of the free radiolabelled ODN or the control IgG–ODN conjugate.

3.3. Conjugate uptake is temperature-dependent

In an attempt to confirm that the antibody–ODN conjugate was entering cells via the intended active receptor-mediated delivery, the temperature-dependency of the uptake process was studied in RBE4 cells. Incubations were carried out at either 37 or 4°C for 1 h. At 4°C, cellular association of the OX-26–ODN conjugate was similar to that of the free ODN at 37°C and is twofold lower than that of the conjugate incubated at 37°C for 1 h (Fig. 3). This suggested that uptake was an active process consistent with an endocytic route of entry.

3.4. OX-26 conjugates enter RBE4 cells via the transferrin receptors

In order to confirm that the OX-26–ODN conjugate follows the transferrin receptor endocytic pathway, competition studies were performed at 37°C for 1 h with up to 100-fold excess of unlabeled free ODN, and 10-fold excess of free control IgG or rat transferrin receptor antibody. The cellular associated radioactivity in the presence of competing rat transferrin receptor antibody was twofold lower than for the corresponding ODN–antibody conjugate. No competitive effect could be detected in the presence of control IgG or free ODN (Fig. 4). Thus, the rat transferrin receptor pathway seems to be implicated in the internalisation of the OX-26–ODN conjugate into these immortalized rat microvessel endothelial cells.

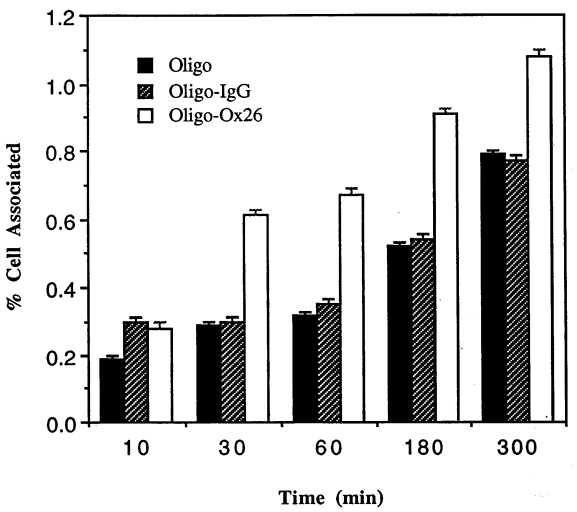

Fig. 2. Cell association of radiolabeled free oligonucleotide (oligo), radiolabeled transferrin receptor antibody–oligonucleotide (oligo–OX-26) and IgG–oligonucleotide (oligo–IgG) conjugates at 37°C in RBE4 cells as a function of time. The same concentration (1 μM) of free oligonucleotide was used as those carried by the antibody–oligonucleotide conjugates. Bars represent standard deviation; $n = 3$. At all time points above 10 min, % cell association of oligo–OX-26 was significantly greater than the free ODN or Oligo-IgG ($p > 0.05$).

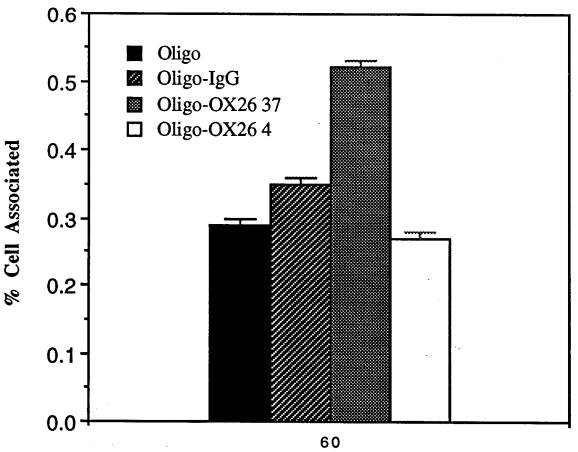

Fig. 3. Influence of the incubation temperature on cellular association of the radiolabeled transferrin receptor antibody–oligonucleotide conjugate in RBE4 cells. Cells were incubated with 1 μM conjugated oligonucleotide for 1 h at 37°C (oligo–OX-26 37) or at 4°C (oligo–OX-26 4). Free oligonucleotide (oligo) and IgG–oligonucleotide (oligo–IgG) conjugate were used as controls and incubated for 1 h at 37°C at the same final concentration of oligonucleotide. Bars represent standard deviation; $n = 3$. The % cell association of oligo–OX-26 at 37°C was significantly greater than that observed at 4°C ($p > 0.05$).

3.5. Conjugate exocytosis: efflux studies

Efflux studies were conducted with the objective of discriminating between the endocytic compartments of free and conjugated ODNs. After 1-h incubation of free or antibody-conjugated ODN at 37°C, the percentage of released radioactivity was assessed considering that the cellular associated radioactivity after 1-h incubation corresponds to 100%. After 5 h of release experiment,

85–100% of respectively free ODN and control IgG conjugated ODN are released in the extracellular medium. In contrast, 37% of the OX-26–ODN conjugate remained associated with the cells (Fig. 5). These results suggest that the OX-26–ODN conjugate is internalised in more profound compartments than the free ODN or the control IgG ODN conjugate.

3.6. Improved cellular accumulation of ODN conjugates upon monensin treatment of cells

Monensin is a Na^+ ionophore which increases the pH of endosomes and lysosomes and also exhibits the property of inhibiting the normal vesicular trafficking via the trans-Golgi network, TGN (Tartakoff, 1983). We, therefore, explored the possibility of using monensin to quantitatively change the internalisation of the OX-26–ODN conjugate. RBE4 cells were treated with 10 μM monensin before and during the incubation with free or conjugated ODN. The treatment with monensin has no effect on the cellular association of free ODN or the control IgG–ODN conjugate, whereas the radioactivity cellular association of the OX-26–ODN conjugate was almost doubled in the presence of monensin (Fig. 6). This result further suggests that the OX-26 conjugate follows the transferrin receptor-mediated endocytic pathway, whose intracellular vesicular trafficking is mediated, at least in part, via the TGN. These data also suggest that free ODNs follow a different pathway and that their intracellular trafficking does not involve TGN.

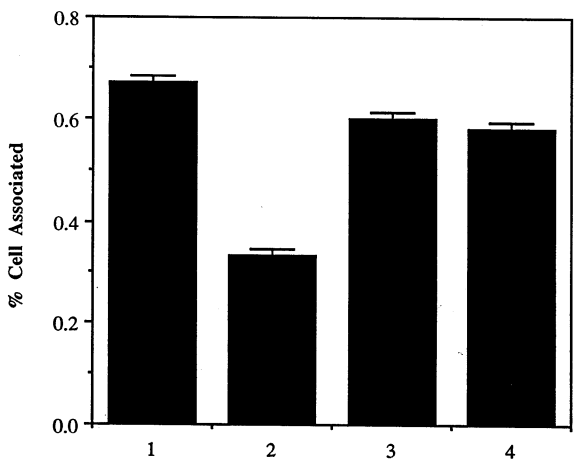

Fig. 4. Influence of competitors on the cellular association of transferrin receptor antibody–oligonucleotide. The percentage of cell association of the radiolabeled transferrin receptor antibody–oligonucleotide conjugate is determined after 1-h incubation at 37°C and compared with those in the presence of 10-fold excess of IgG or transferrin receptor antibody and in the presence of 100-fold excess of free oligonucleotide. Lane 1 is the radiolabeled transferrin receptor antibody–oligonucleotide conjugate. Lane 2 shows the competition with the transferrin receptor antibody. Lanes 3 and 4 show incubations with free oligonucleotide and IgG respectively. The non-labeled competitors were added 15 min prior to incubation with the radiolabeled transferrin receptor antibody–oligonucleotide conjugate. Bars represent standard deviation; $n = 3$. The reduction in % cell association of oligo–OX-26 upon competition with transferrin receptor antibody was statistically significant ($p \geq 0.05$).

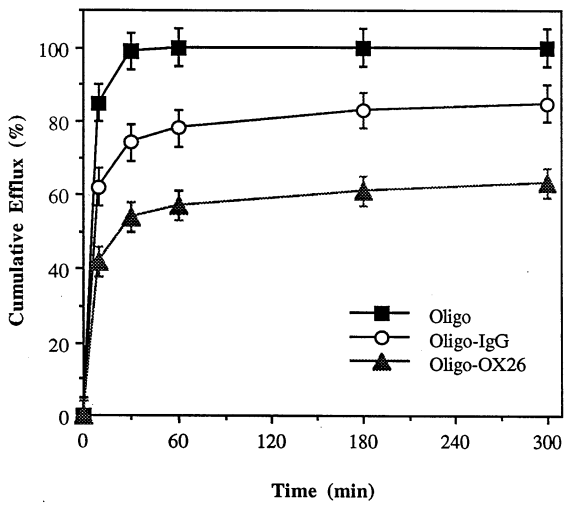

Fig. 5. Efflux studies. The release of free oligonucleotide (oligo), oligonucleotide conjugated with IgG (oligo–IgG) or with transferrin receptor antibody (oligo–OX-26) is studied after 1 h incubation at 37°C with RBE4 cells over 5 h. Bars represent standard deviation; $n = 3$.

4. Concluding remarks

The RBE4 cells, which we used to assess the cellular association of free and conjugated ODNs, are reported to constitute a good, yet simple model for the BBB (Abbott et al., 1995). Our results show that the uptake of antisense ODNs

into this in vitro model of the BBB can be significantly improved upon conjugation to an anti-transferrin receptor antibody, OX-26. Delivery of ODNs by transferrin receptor-mediated uptake may be further enhanced by manipulating the intracellular trafficking of ODN conjugates with chemical agents such as monensin. These data now form the basis for further studies characterising the CNS delivery of ODNs across the BBB, e.g. studying their transcytosis across RBE4 cells grown on polycarbonate filters. Although the present study highlights the importance of modulating cell trafficking of ODNs as a potential therapeutic approach, the pharmacological activity of antisense ODNs will ultimately be governed by their ability to reach the mRNA target located in the cytoplasm. This further suggests the need to develop coupling strategies (e.g. disulfide-bridged linkages instead of thioether linkages) that would enable the liberation of the ODN from its carrier within the reducing environment of the target cell. Such studies are planned.

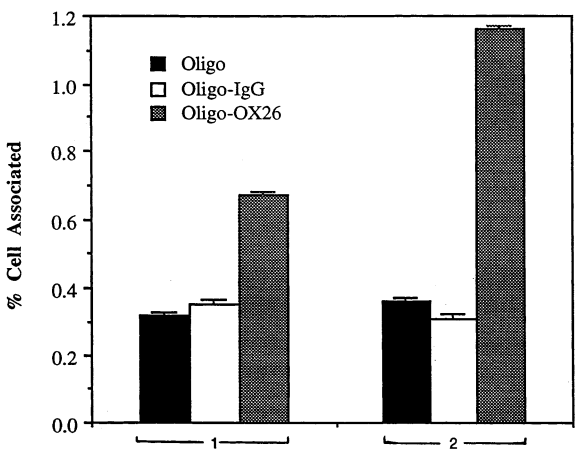

Fig. 6. The effect of monensin treatment on the cellular association of free or conjugated oligonucleotide in RBE4 cells. The percentage of cell association of radiolabeled free oligonucleotide (oligo) and transferrin receptor antibody–oligonucleotide (oligo–OX-26) or IgG-oligonucleotide (oligo–IgG) conjugates were compared when incubated 1 h at 37°C with and without 10 μM monensin. The monensin was added 15 min prior to the 1-h incubation. Lanes 1 and 2 show cell association without and with monensin, respectively. Bars represent standard deviation; $n = 3$. The % cell association observed in the presence of monensin was significantly greater than in the absence of monensin treatment ($p > 0.05$, Students t-test).

Acknowledgements

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