Improved Cellular Delivery of Antisense Oligonucleotides Using Transferrin Receptor Antibody-Oligonucleotide Conjugates

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Received February 20, 1995; accepted April 30, 1995

KEY WORDS: antisense oligonucleotides; transferrin receptor antibody; cell uptake; receptor-mediated delivery; nucleic acids; antibody-oligonucleotide conjugates.

INTRODUCTION

Antisense oligodeoxynucleotides have been used to inhibit target mRNA translation both *in vitro* and *in vivo* and are currently being evaluated clinically as therapeutic agents for the treatment of human malignancies and viral diseases such as AIDS [1]. However, major limitations in the effective use of antisense nucleic acids as inhibitors of gene expression in the clinic include their poor biological stability [2] and their inefficient delivery to target cells [3]. Unmodified phosphodiester and phosphorothioate oligodeoxynucleotides are thought to enter living cells via a combination of receptor-mediated and/or fluid-phase endocytosis [4–6] whereas methylphosphonate oligodeoxynucleotides are reported to enter cells predominantly by fluid-phase endocytosis [7]. These natural routes of cellular entry for oligodeoxynucleotides are highly inefficient, typically yielding very low intracellular concentrations. Thus, the potential utilization of a more efficient internalization process for oligodeoxynucleotides has been extensively sought [3].

The transferrin receptor which efficiently transports iron-bearing transferrin molecules into cells by receptor-mediated endocytosis, represents a useful carrier by which oligonucleotides may be potentially internalized. This receptor, a 180 kDa dimeric transmembrane glycoprotein, has been reported to efficiently internalize DNA, oligonucleotides and low molecular weight drugs that have been complexed with its natural ligand, transferrin [8,9]. However, when administered *in vivo*, transferrin-containing conjugates or complexes are likely to be in competition with the uptake of the natural transferrin protein and could potentially compromise normal iron uptake into cells. An alternative approach would be to use monoclonal antibodies to the transferrin receptor that recognise and bind to regions other than the transferrin binding site of the receptor and thus, would not compete for the natural ligand. The cellular delivery of peptides and small molecular weight drugs with such transferrin receptor antibodies (e.g. OX-26) has been demonstrated previously [10]. In this study, we have examined the possibility of conjugating oligonucleotides to a human monoclonal transferrin receptor-antibody (RVS-10) for the improved delivery of nucleic acids to human cells.

METHODS

Synthesis and 3′-end [^{32}P]-Radiolabelling of Oligonucleotides

Antisense phosphodiester oligonucleotides and 5′-end C_6 thiol-modified oligonucleotides were synthesised on an automated DNA synthesiser (Model 392, Applied Biosystems, Warrington, UK) using standard phosphoramidite chemistry (0.2 μM scale). As model compounds, a 20 mer sequence antisense to the 3′-splice site of the *tat* gene in HIV-RNA (3′ ACA CCC AAT TCT GAA AAT GG 5′) and 19-mer sequence (minus the 3′-terminal adenosine present in the 20 mer) were synthesized with and without a 5′-end trityl protected thiol group using the C_6-Thiol modifier (Cruachem United Kingdom). The thiol protecting group was subsequently removed with silver nitrate/dithiothreitol (Cruachem User instructions No. 005, March 1994) to yield the free thiol before conjugation with derivatized monoclonal antibodies (see below). The 19 mer was subsequently converted to a 20-mer sequence upon 3′-end [^{32}P] radiolabelling with alpha [^{32}P] ddATP and terminal transferase according to the manufacturer's protocol (Boehringer Manheim). The radiolabelled oligonucleotides were then purified by native 15% polyacrylamide gel electrophoresis. The concentration of the eluted oligonucleotide solution was determined by U.V. absorption at 260nm assuming that a value of 1OD unit was equivalent to 29.4μg [11].

Derivatization of Monoclonal Antibodies with the Heterobifunctional Cross-Linker, SMCC

To a solution of mouse-anti-human transferrin receptor antibody (Clone RVS-10 (IgG1 subclass); Biogenesis, UK) or a control mouse-anti-human IgG (2–4 mg) in 2.5ml of 50mM phosphate buffer (pH 8.0) was added 0.05M of the heterobifunctional cross linker, succinimidyl 4-(maleimidomethyl) cyclohexane-1-carboxylyate (SMCC) in dimethylformamide (DMF) solution. The reaction mixture was incubated for 90 mins at room temperature in the dark. Excess SMCC was removed by gel filtration on Sephadex G-25 (1.6cm × 20 cm column, Pharmacia) in 50mM phosphate buffer (pH 6.0), containing 0.1 M NaCl and 5mM EDTA. The resulting concentration of the antibodies was determined by measuring absorbance at 280nm (1mg/mL = 1.4 OD units) [12].

Conjugation of Thiol-Modified Oligonucleotides to SMCC-Modified Monoclonal Antibodies

Deprotected 5′-end thiol-modified oligonucleotides bearing a 3′-end [^{32}P] radiolabel (310 μg) were dissolved in 1ml of the reaction buffer at pH 7.0 (50 mM phosphate buffer, 0.1 M NaCl and 5 mM EDTA) which had been thoroughly de-gassed with nitrogen. To this was added 750μg of the SMCC-derivatized monoclonal antibody (either transfer-

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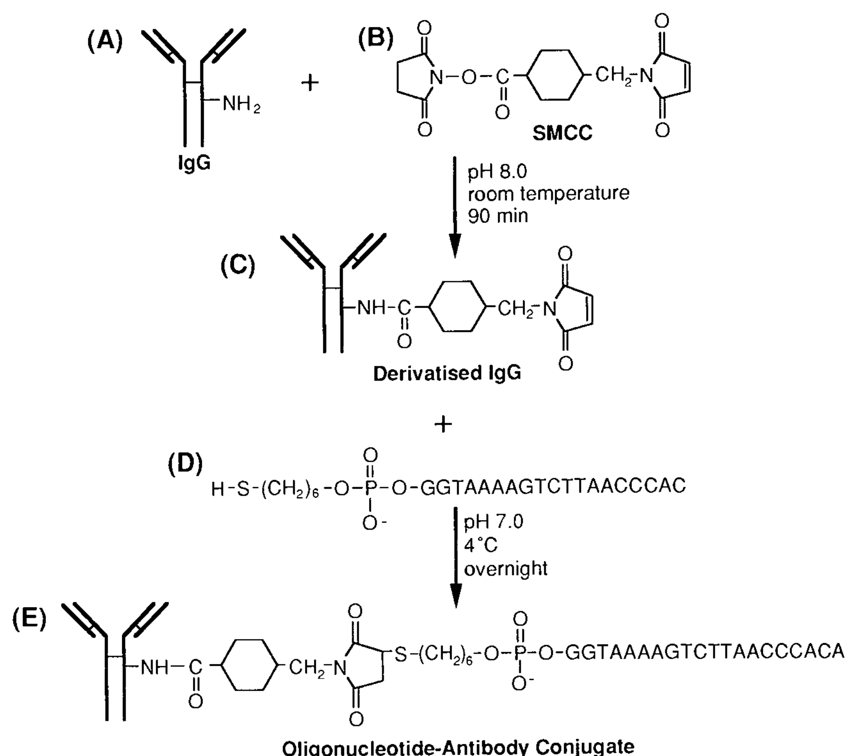

Scheme 1. Synthesis of monoclonal antibody-oligonucleotide conjugates. This synthetic procedure was used to manufacture antisense oligonucleotide conjugates with both the human transferrin receptor-antibody (IgG_1 subclass, clone RSV-10) and the human IgG_1 antibody (see Methods for details of reactions).

rin receptor antibody or a control human IgG) in 1ml of the same reaction buffer and left overnight at 4°C. In order to block any unreacted maleimide groups introduced by the SMCC on the antibody, the conjugate was reacted with 0.01M aqueous solution of 2-mercaptoethylamine for 2 h at room temperature. Finally, the conjugate was purified by gel filtration on Sephadex G-25 (1.6cm × 20 cm column, Pharmacia) and eluted in 50mM phosphate buffer (pH 7.4), containing 0.25 M NaCl. The first 1–2 ml fraction contained the purified conjugate as verified by 10% SDS-PAGE analysis under non-reducing conditions. The number of oligonucleotide molecules conjugated to the transferrin receptor-antibody was quantified spectrophotometrically by determination of the absorbance of the conjugate at 260nm and at 280nm. The degree of conjugation was then derived from the A260/A280 ratio as described by Kuipers *et al.* [12]. During the above synthetic reactions, there was no evidence of oligonucleotide degradation and the overall yields of the antibody-oligonucleotide ranged from approximately 45–65%.

Cell Culture and Histochemical Staining of Transferrin Receptors

The human glioblastoma cell line, U87-MG, and the human endothelial cell line, ECV304, were purchased from the European Cell Culture Collection. Both cell lines were grown in D-MEM (Gibco, United Kingdom) supplemented with 10% foetal calf serum. The expression of the transferrin receptor on the cell surface of these cell lines was established by histochemical staining. The primary human transferrin antibody (Biogenesis, United Kingdom) was detected with VECTASTAIN ELITE ABC Kit (Vector Laboratories, California, USA) as described in the manufacturer's protocol.

Cell Uptake Studies

For uptake experiments, 1 × 10^5 cells/well (24-well plate) were incubated with either 3′-[^{32}P] labelled oligonucleotide-antibody conjugates or radiolabelled oligonucleotides alone in serum-free D-MEM medium. When competitors were used, these were added 15 mins prior to addition of labeled antibody-oligonucleotide conjugates. After the desired incubation times (30 min–5h), the medium was removed and cells washed 3 times with 0.5 ml PBS. Cells were then lysed with 1–3% SDS and cell-associated radioactivity was determined by liquid scintillation counting. Uptake studies were performed either at 4°C or 37°C. In some experiments, in order to strip off surface bound oligodeoxynucleotides, 0.4 M acetate buffer (pH 3.2) was used instead of PBS as the washing solution in the final wash prior to cell lysis. Low pH washing has been reported to remove almost 95% of cell surface bound ligands [13].

RESULTS AND DISCUSSION

Conjugate Synthesis and Characterization

It was our aim to evaluate transferrin-receptor antibody (TRA)-oligonucleotide conjugates for the improved delivery of antisense oligonucleotides to living cells. Scheme 1 sum-

marizes the synthesis of the antibody-oligonucleotide conjugates. A maleimido group was introduced onto the antibody (A) using the heterobifunctional cross-linker, SMCC (B), to produce a derivatized protein (C). This was then reacted with a free thiol group present on a derivatized-oligonucleotide (D) to form the antibody-oligonucleotide conjugate (E) (See Methods for details of synthesis). This synthetic procedure was used for producing both the TRA-oligonucleotide and for the control non-specific human IgG-oligonucleotide conjugates. To confirm their constitution, the purified conjugates (see Methods) were characterized in terms of their susceptibility to degradation by DNAse 1 and by Proteinase K and their relative mobilities on 10% SDS-PAGE gels (see Figure 1). The gel mobilities of the TRA-oligonucleotide and human IgG-oligonucleotide conjugates were similar, both migrating with an apparent molecular weight of around 160 kDa. Lane C shows that simple incubation of labelled oligonucleotide with antibody under reaction conditions exhibited a band corresponding to the free oligonucleotide. This indicated that the conjugate was not simply a result of the oligomer adsorbing onto the antibody but implied interaction by covalent coupling as suggested by scheme 1. In an attempt to confirm the presence of the nucleic acid and protein components of the conjugate, enzyme digestion studies were performed. Partial digestion of the conjugate with DNase 1 (lane D) resulted in the monomeric ATP, a product which is characteristic of oligonucleotide digestion by the enzyme and indicated that the conjugate had an oligonucleotide component. To verify the protein component of the conjugate, partial digestion with Proteinase K (lane E) was performed. In this case, a band exhibiting a mobility similar to the free oligonucleotide (Lane B) was observed indicating release of the oligomer from the digested protein. The SDS-PAGE data were consistent with the proposed antibody-oligonucleotide synthetic product shown in scheme 1. Determination of the conjugation ratio according to the method of Kuipers *et al.* [12], suggested that our synthesis resulted in the coupling of, on average, approximately 10 molecules of oligonucleotide per molecule of antibody.

Cellular Uptake

The cellular uptake properties of the transferrin receptor antibody-oligonucleotide conjugate were evaluated in two cell lines; a glioma cell line U87-MG and an endothelial cell line, ECV304. Histochemical staining confirmed that both cells expressed the TRA receptor to similar levels (data not shown). In both cell lines, cellular association of the TRA-oligonucleotide conjugate was approximately 3-fold higher than the free oligonucleotide at incubation periods between 2 and 5 hours and approximately 2-fold higher than a non-specific IgG-antibody-oligonucleotide conjugate after 2 h in ECV304 cells (Figures 2A and 2B).

It should be noted, however, that cellular association of conjugate and oligonucleotides was generally higher in glioma cells than that observed with the endothelial cells when similar extracellular concentrations of oligonucleotide or conjugate were used in each cell type. These data further highlight the fact that cell association and uptake of oligonucleotides is dependent on cell type [4–7]. These differences in basal levels of oligonucleotides uptake between the two cell types used here cannot be explained in terms of either differences in transferrin receptor expression levels, which were similar in both cell lines, or to the stability of antibody-oligonucleotide conjugate (data not shown). As evidenced by PAGE analyses of cell culture supernatants, the oligonucle-

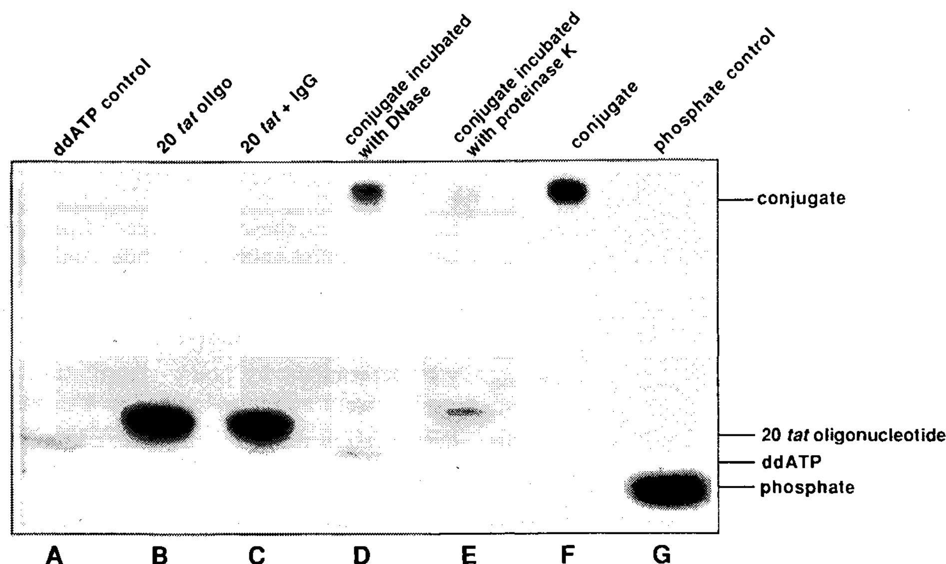

Fig. 1. Characterization of the transferrin receptor antibody-oligonucleotide conjugates. The conjugates and the various control samples were run on a 10% SDS-PAGE gel. Lane A is the free label, alpha [^{32}P] ddATP, used for the 3′-end radiolabelling of oligonucleotides. Lane B is 3′-end [^{32}P] radiolabelled 20-mer *tat* oligonucleotide. Lane C is a physical mix of the radiolabelled 20-mer *tat* oligonucleotide and the non-derivatized transferrin receptor antibody subjected to the same reaction conditions as shown in Scheme 1. Lane D is TRA-oligonucleotide conjugate incubated with DNase 1. Lane E is TRA-oligonucleotide conjugate incubated with Proteinase K. Lane F is the TRA-oligonucleotide conjugate and Lane F is the free phosphate, [$^{32}P_i$].

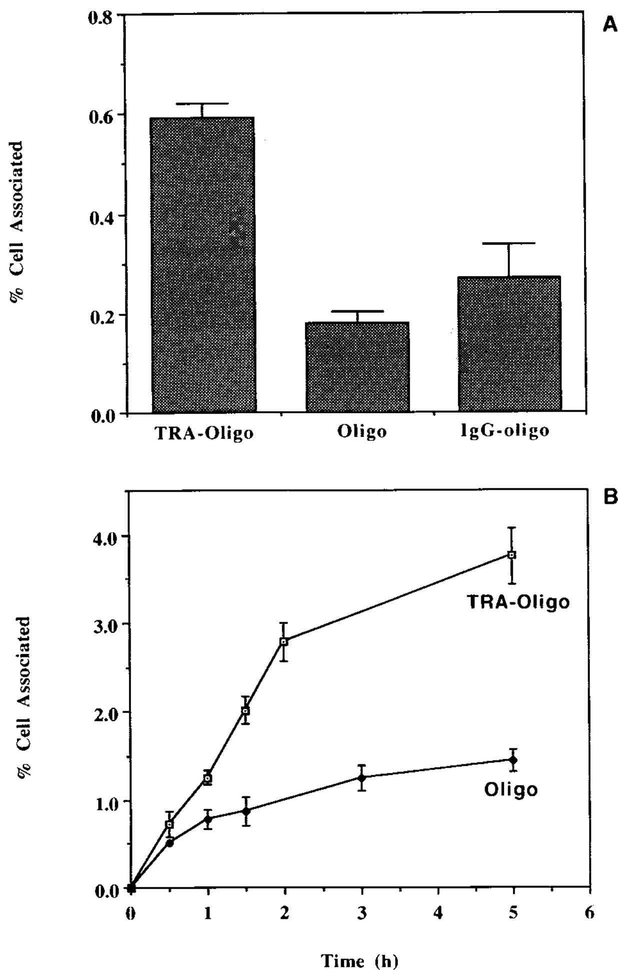

Fig. 2. Cell association of transferrin receptor antibody-oligonucleotide (TRA-oligo) conjugates at 37°C. (A) Cell association in ECV304 cells after 2h incubation in serum-free D-MEM medium and compared to free 20-mer tat oligonucleotide (oligo) and to the corresponding non-specific human IgG-oligonucleotide conjugate (IgG-oligo) as a control. (B) Cell association of TRA-oligo conjugate and the free oligonucleotide in U87-MG glioma cells as a function of time. In all cases, the same concentrations of free oligonucleotide were used as those carried by the antibody-oligonucleotide conjugate (see Methods for experimental details). Bars represent standard deviations; n = 3.

otide conjugate remained stable in the serum-free culture conditions used for both cell types (data not shown). It is conceivable that variations in cell association and uptake may be explained by differences in the levels of expression of oligonucleotide binding proteins, such as those described by Loke *et al.* [4] and by Yakubov *et al.* [5]. However, these were not investigated in the present study.

The cellular association of the TRA-oligonucleotide conjugate and the free oligonucleotide was temperature-dependent (Figure 3A). In U87-MG cells, a 2-fold association of the conjugate was observed at 37°C than at 4°C after a 1h incubation. In the case of the free oligonucleotide, a 7-fold difference in cell association was observed between the two incubation temperatures in U87-MG cells (see Figure 3A).

The data in Figures 2 and 3A indicate that the TRA-oligonucleotide conjugate exhibits greater cell surface binding than the free oligonucleotide. In order to assess what proportion of the cellular associated TRA-oligonucleotide was actually internalized by U87-MG cells, acid washing with acetate buffer at pH 3.2 was undertaken (see figure 3B). This technique is thought to remove approximately 95% of surface-bound ligands [13] and has previously been used to remove surface bound oligonucleotides from other cell lines [7]. Acid washing of U87-MG cells following incubation of the conjugate for 1 h at 37°C removed approximately 60% of the cell associated radioactivity suggesting that almost 40% of the TRA-oligonucleotide conjugate had been internalized (Figure 3B).

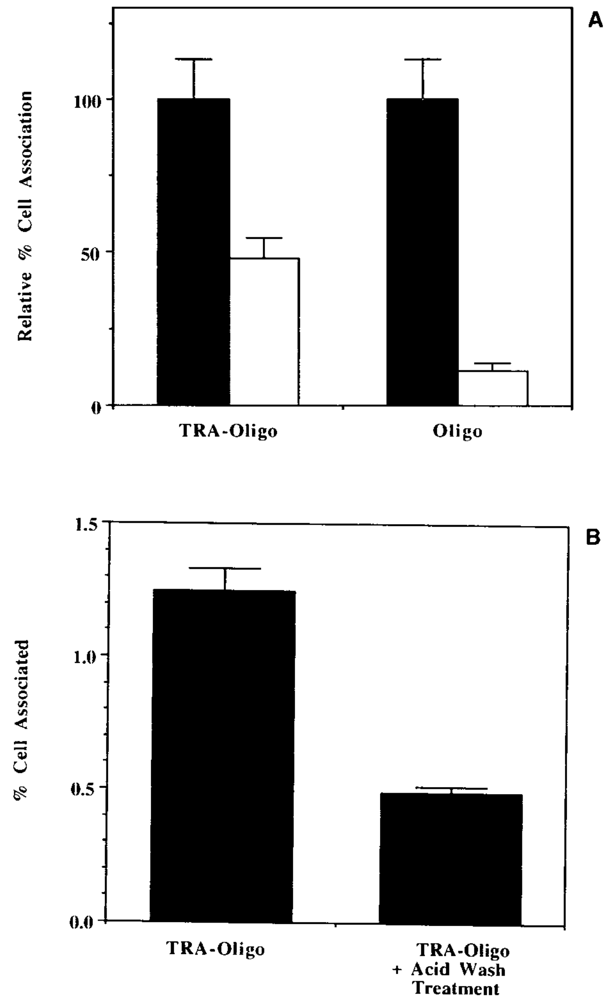

Fig. 3. (A) The influence of incubation temperature on cellular association of the radiolabelled TRA-oligonucleotide (TRA-oligo) conjugate and 3′-end [^{32}P]-radiolabelled 20-mer *tat* oligonucleotide (oligo) in U87-MG cells after 1h. Black boxes represent 37°C data and the open (white) boxes are data at 4°C. The data is presented as the relative % cell association where cell association values at 37°C were taken as 100% for both the TRA-oligonucleotide conjugate and the free oligonucleotide. In all cases, the same concentrations of free oligonucleotide were used as those carried by the antibody-oligonucleotide conjugate (see Methods for experimental details). (B) The effect of acid-washing on cellular association of TRA-oligo conjugate in U87-MG cells after 1h at 37°C. Acid washing was performed as described in the Methods. Bars represent standard deviations; n = 3.

To further investigate whether the TRA-oligonucleotide conjugate was taken up by cells via the transferrin receptor-mediated pathway or by the pathway utilised by free phosphodiester oligonucleotides, we performed competition studies. Figure 4A shows that competition by a 10-fold excess of transferrin receptor antibody in ECV304 cells was sufficient to reduce cellular association to the levels observed for the non-specific IgG-antibody-oligonucleotide-conjugate. Transferrin receptor antibody was also able to efficiently compete for uptake of the TRA-oligonucleotide conjugate apparently in a dose-dependent manner in U87-MG cells (Figure 4B). In this case, a 1:1 ratio of unlabelled TRA:labelled TRA-oligonucleotide conjugate resulted in approximately a 50% reduction in cellular association. Incubation with a 100-fold excess of the free unlabelled phosphodiester oligonucleotide produced a modest (less than 20%) reduction in cellular association whereas phosphorothioate oligonucleotides (150-fold excess) had little or no effect on the cellular association of the conjugate. These results suggest that the transferrin receptor-antibody oligonucleotide conjugate is entering cells predominantly by the intended transferrin receptor pathway with very little, if any, entering cells via recognition of the oligonucleotide component of the conjugate.

We have demonstrated that oligonucleotides can be conjugated to the transferrin receptor antibody for the improved cellular delivery of antisense oligonucleotides. A three- to four-fold enhancement in cell-association of oligonucleotide was achieved when using the conjugate in our two cell lines. These data, with the antibody-oligonucleotide conjugate, correlate well with studies using transferrin-polylysine-oligonucleotide complexes where a 4-fold increase in the antiproliferative effects of c-myb antisense oligonucleotides were observed upon complexation [9]. However, further improvements in cellular delivery may be possible in cells that exhibit much higher expression of the transferrin receptor as has been observed for some tumour cells [9]. In particular, TRA-oligonucleotide conjugates may be useful for delivering nucleic acids across the blood-brain barrier (BBB) as the microvessel endothelial cells constituting the BBB have a high abundance of the transferrin receptor [14]. Free oligonucleotides are effectively excluded from the brain because fluid-phase endocytosis, a normal cellular entry pathway for oligonucleotides [3], is severely attenuated in microvessel endothelial cells of the BBB. However, the high abundance coupled with the demonstrated transcytotic capability of transferrin receptors [10,14] may facilitate receptor-mediated delivery of oligonucleotides across the BBB and into the brain. However, as DNA oligonucleotides are rapidly digested in serum [2], it may be necessary to use oligonucleotides that are less-sensitive to nuclease digestion, such as phosphorothioate end-protected oligodeoxynucleotides or methylphosphonate analogues. In this study we have used a stable thioether linkage for conjugating oligonucleotides to the transferrin antibody, however, for the effective delivery of oligonucleotides it may be desirable to use a cell-labile disulphide linkage which would facilitate oligomer release from the complex in the reducing environment of the target cells.

It should be noted that uptake of conjugates by receptor-mediated endocytosis may still result in oligonucleotide accumulation into endosomal and lysosomal vesicles within the cytosol of the target cell. Rapid escape from such acidic compartments will be necessary to exert an intracellular biological effect [3] and to avoid oligonucleotide loss from the cell through exocytosis [6] or by lysosomal degradation (Akhtar *et al*, submitted). The intracellular trafficking of antibody-oligonucleotide conjugates is currently under investigation. However, should vesicular localization become a major limitation for antibody-conjugates, then efflux from these vesicular compartments may be facilitated by the inclusion of pH-sensitive fusogenic molecules (e.g. influenza virus HA2 peptides) into the conjugate as these are thought to aid

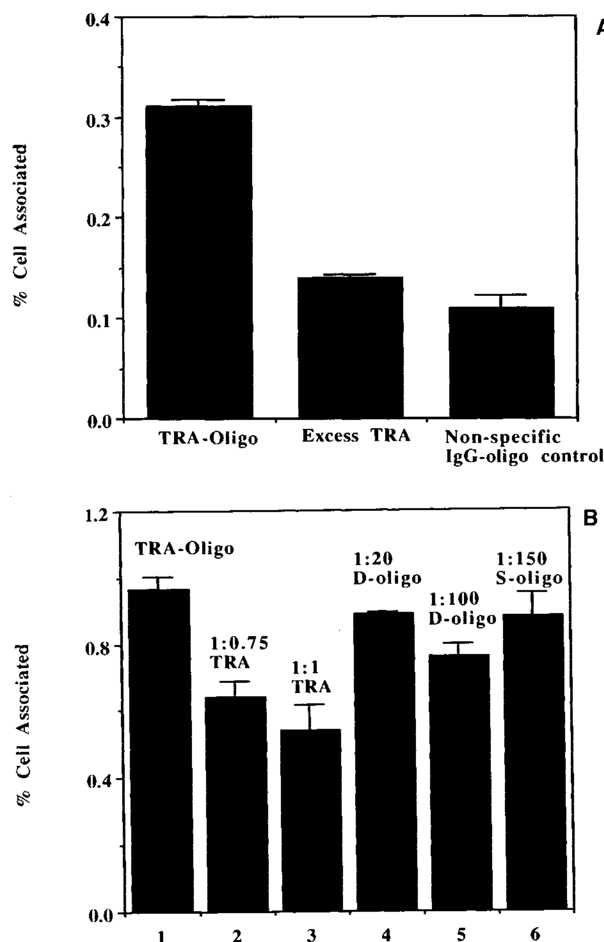

Fig. 4. The influence of competitors on the cellular association of TRA-oligo conjugate. (A) Competition in ECV304 cells. The % cell association of the control radiolabelled TRA-oligo conjugate is compared with that of the non-specific IgG antibody-oligo control and when a 10-fold excess of transferrin receptor antibody was added to the TRA-oligo (Excess TRA). (B) Competition in U87-MG cells. Lane 1 is the radiolabelled TRA-oligo conjugate. Lanes 2 and 3 show competition with unlabelled transferrin receptor antibody at the stated ratios (1:0.75 and 1:1 respectively). Lanes 4 and 5 show incubations with a 20-fold and 100-fold excess unlabelled 20 mer *tat*-phosphodiester oligonucleotides (D-oligo) respectively. Lane 6 shows incubation with a 150-fold excess of a 20-mer phosphorothioate oligonucleotide (S-oligo). All competition experiments were performed at 37°C after 1h incubation. The non-labelled competitor was added 15 mins prior to incubation with the radiolabelled TRA-oligo conjugate. Bars represent standard deviations; n = 3.

endosome-to-cytosol transfer of oligonucleotides and DNA complexes [15].

ACKNOWLEDGMENTS

We would like to thank Ramila Patel for assistance with the cell culture studies. This work was supported in part by grants from the MRC AIDS directed programme, the Cancer Research Campaign and the Nuffield Foundation.

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