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Carbohydrate receptor-mediated gene transfer to human T leukaemic cells

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The mucin-type carbohydrate Tn cryptantigen (GalNAcα1-O-Ser/Thr, where GalNAc is *N*-acetyl-D-galactosamine) is expressed in many carcinomas, in haemopoietic disorders including the Tn syndrome, and on human immunodeficiency virus (HIV) coat glycoproteins, but is not expressed on normal, differentiated cells because of the expression of a Tn-processing galactosyltransferase. Using Jurkat T leukaemic cells which express high levels of Tn antigen due to deficient Tn galactosylation, we have established the Tn antigen-mediated gene transfer and demonstrate the considerable efficiency of this approach. We used poly(L-lysine) conjugates of the monoclonal antibody 1E3 directed against the Tn antigen to deliver the luciferase and β-galactosidase reporter genes to Jurkat cells by receptor-mediated endocytosis. Addition of unconjugated 1E3 reduced transfection efficiency in a concentration-dependent manner and incubation with free GalNAc abolished DNA transfer completely, indicating that gene delivery is indeed mediated by the Tn antigen. Pre-treatment of Jurkat cells with *Vibrio cholerae* sialidase, which uncovers additional Tn antigens, resulted in an improvement of gene transfection. Both human and chicken adenovirus particles attached to the DNA/polylysine complex strongly augmented transgene expression. When the β-galactosidase (lacZ) gene was delivered to Jurkat cells by Tn-mediated endocytosis, up to 60% of the cells were positive in the cytochemical stain using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) as a chromogenic substrate. The efficiency of the transferrin receptor-mediated DNA uptake into Jurkat cells was comparatively low, although these cells were shown to express considerable amounts of transferrin receptor. We show here that a mucin-type carbohydrate antigen mediates highly efficient DNA uptake by endocytosis into Jurkat T cells. This method represents a 50-fold improvement of Jurkat cell transfection efficiency over other physical gene transfer techniques. Specific gene delivery to primary cancer cells exhibiting Tn epitopes may especially be desirable in immunotherapy protocols.

Key words: adenovirus/endocytosis/gene transfer/T cell/Tn antigen

Introduction

The success of gene therapy depends on efficient methods to deliver genes to somatic cells, including T lymphocytes (Miller, 1992). In addition to retroviral systems which are used in approved clinical trials (Rosenberg *et al.*, 1990), alternative physical methods have been elaborated and shown to be efficient. In a very promising approach designated as transferrinfection, DNA is bound by a poly-L-lysine (pL) transferrin (Tf) conjugate and delivered to the cell interior by transferrin receptor (TfR)-mediated endocytosis (Cotten *et al.*, 1990, 1992; Wagner *et al.*, 1990, 1991a,b, 1992). However, while the TfR-mediated gene transfer proved to be highly efficient for many cell types, and in particular for erythroid K562 cells, T lymphoid cells including Jurkat appeared to be refractory (Cotten *et al.*, 1993a), calling for different strategies. In earlier experiments, glycoproteins were used to target DNA to the lectin-like asialoglycoprotein receptor expressed on hepatocytes (Wilson *et al.*, 1992). Conversely, it should be possible to use carbohydrate-specific reagents to target DNA to cells exhibiting these carbohydrate epitopes. An obvious advantage of cell surface carbohydrates, in particular of the mucin type, as receptors for gene transfer experiments is their clustered expression, resulting in high surface densities (Carraway and Hull, 1991). In fact, a protein-specific monoclonal antibody (mAb) recognizes a single epitope on a cell surface glycoprotein, whereas an anti-carbohydrate mAb may bind to multiple receptors attached to the same protein. Thus, we decided to target a mucin-type carbohydrate antigen to transfect Jurkat T cells.

For unknown reasons, Jurkat cells lack *O*-glycan-specific β1,3 galactosyltransferase (Gal-T) activity and therefore express the Tn cryptantigen. The Tn antigen is the initial carbohydrate structure formed in the biosynthesis of O-linked carbohydrates of the mucin type, also referred to as *O*-glycans (Schachter and Brockhausen, 1989). In normal differentiated cells, the Tn antigen is cryptic due to carbohydrate chain elongation or branching. Loss of β1,3Gal-T, the Tn-processing enzyme, leads to exposure of the Tn antigen which may be partially sialylated to form the sialosyl-Tn antigen (NeuAcα2-6GalNAcα1-O-Ser/Thr, where GalNAc is *N*-acetyl-D-galactosamine) (Kjeldsen *et al.*, 1989; Blumenfeld *et al.*, 1992). Deficiency of β1,3Gal-T resulting in Tn/sialosyl-Tn expression is known to be associated with oncogenic transformation (Springer, 1984; Singhal and Hakomori, 1990). Expression of these antigens is particularly well established in breast and colon cancer (Itzkowitz *et al.*, 1989; Springer, 1989), but also occurs in the context of haemoproliferative disorders (Roxby *et al.*, 1992), including acute lymphoblastic T-cell leukaemia (Piller *et al.*, 1990). Moreover, Tn/sialosyl-Tn expression was also found in idiopathic Tn syndrome (Thurnher *et al.*, 1992, 1993a), a condition considered to be a pre-leukaemic state. Finally, the Tn epitope has also been detected on gp160 and gp120 of human immunodeficiency virus (HIV) (Hansen *et al.*, 1991). mAb against the Tn antigen neutralized infection with

cell-free virus and blocked syncytium formation. In contrast, normal adult cells lack detectable Tn antigen expression. Thus, the Tn antigen is a useful histological marker which delineates malignant tissue, suggesting that Tn-specific mAb or lectins (Thurnher *et al.*, 1993b) may have therapeutic potential.

To test Tn antigens as receptors for gene delivery, we have used polylysine conjugates of the anti-Tn mAb 1E3 to transfect the luciferase or the β-galactosidase marker gene to Tn+ Jurkat T cells which were previously shown to bind 1E3 (Thurnher *et al.*, 1992). Specific and efficient Tn-mediated gene transfer to Jurkat T cells could be performed.

Results

The Tn antigen is expressed on Jurkat cells at high density

In a preliminary analysis, we compared the levels of surface expression of the TfR, the target of the transferrinfection (Wagner *et al.*, 1992), and of the Tn antigen. The flow cytometric analysis depicted in Figure 1 revealed that the surface density of the Tn antigen was much higher than that of the TfR on Jurkat cells (Figure 1, compare B with C, see also Table I). However, the level of TfR density on Jurkat cells was surprisingly high when compared to erythroid K562 cells (Figure 1A and B). In K562 cells, TfR expression can be upregulated by treatment with desferrioxamine (Mattia *et al.*, 1984) (see also Figure 1A and Table I). In addition, Jurkat cells were also stained for the CD43 antigen which is a major Tn antigen-bearing surface protein on Jurkat cells (Piller *et al.*, 1990). Figure 1D confirms the expression of CD43 on Jurkat T cells. The CD43 mAb DF-T1 used for this labelling is, like most of the mAbs recognizing CD43, sialic acid dependent (Borche *et al.*, 1987). Thus, pre-treatment with *Vibrio cholerae* sialidase (VCN) abolished DF-T1 binding (Figure 1D, left peak).

mAb 1E3 mediates gene transfer to Jurkat cells

Previous studies on the receptor-mediated DNA transfer revealed that one function of the polylysine is the condensation of the DNA to be transfected into small toroid structures small enough to be engulfed into coated pits (Wagner *et al.*, 1991b). Thus, in preliminary experiments the optimal ratio of 1E3pL and pCLuc had to be determined. For this purpose, decreasing amounts of conjugate were mixed with a constant amount of DNA and transfected into Jurkat cells in the presence of 100 μM chloroquine. Figure 2 demonstrates that 5 μg of 1E3pL and 6 μg of pCLuc resulted in the highest transfection efficiency (compare 1–5). Therefore, in all further experiments a 5:6 ratio of conjugate to DNA was used. Despite considerable TfR expression (Figure 1B), transferrinfection of Jurkat cells was 40 times less efficient than Tn-mediated gene delivery (Figure 2, no. 6). In the absence of chloroquine, transgene expression was completely prevented (Figure 2, no. 7).

The gene transfer is specifically mediated by the Tn antigen

Flow cytogram C in Figure 1 demonstrated that binding of 1E3 to Jurkat cells was significantly enhanced by enzymatic desialylation with VCN, suggesting that additional Tn epitopes are unmasked. We have therefore tested the influence of VCN pre-treatment of Jurkat cells on the Tn-mediated gene transfer. Figure 3 shows a 19.5% increase in luciferase activity induced by VCN (compare 1 and 2). However, while the fluorescence intensity doubled after VCN in the flow cytometric analysis, the improvement of transfection efficiency was relatively weak. This may be due to the removal of negative charge carried by sialic acid which may otherwise bind electrostatically the polycation 1E3pL. Therefore, VCN treatment certainly exposes additional Tn antigens, as shown in the FACS analysis presented in Figure 1, but also reduces the electrostatic capacity of the cell to bind 1EpL. Thus, the net effect of enzymatic desialylation is only a 19.5% increase in transgene expression.

The specificity of the DNA transfer was further assessed by incubation with either unconjugated 1E3 or in the presence of free GalNAc. Prior addition of 50 or 100 μg of unconjugated 1E3, which represents a 12.5- and 25-fold excess of mAb, respectively, to the transfection medium (2.5 ml) resulted in a 10 and 33% reduction of luciferase activity (Figure 3, nos 3 and 4). This relatively weak inhibition may be explained by a clearing of the unconjugated 1E3 from the medium by endocytosis during the 4 h transfection process, followed by binding and internalization of DNA-loaded 1E3pL. However, when the transfection was set up in the presence of free GalNAc at 30 mM, 1E3 binding and thus DNA uptake was almost completely absent (Figure 3). These data indicated that the gene transfer was indeed mediated by the Tn antigen.

Adenovirus augments Tn-mediated gene transfer to Jurkat cells

The endosome-disrupting activity of viruses which gain entry to the cell via the endocytotic pathway has been described previously (Blumenthal *et al.*, 1986; Otero and Carrasco, 1987; Christiano *et al.*, 1993). The use of either free adenovirus particles or adenovirus particles included in DNA/pL complexes has been shown to increase transgene expression significantly by improving survival of the DNA during intracellular transport. We have therefore investigated whether adenovirus particles also augment Tn-mediated gene delivery to Jurkat cells. For this purpose, we used either human adenovirus H5*dl*1014 (Bridge and Ketner, 1989) or the chicken adenovirus CELO (chicken embryo lethal orphan) (Cotten *et al.*, 1993b). An advantage of the chicken adenovirus would be its inexpensive production and its safety since it is completely non-infectious in humans. In addition, CELO virus displayed far less cellular toxicity, allowing stable tansfection (Cotten *et al.*, 1993b). Figure 4 demonstrates the effect of adenovirus particles on Tn-mediated gene delivery. As expected, human adenovirus strongly augmented DNA transfection (Figure 4, no. 4; note that all adenovirus experiments were carried out with 5 μg 1E3pL and 6 μg pCLuc, while the chloroquine experiment was done with 15 μg 1E3pL and 18 μg pCLuc). In addition, CELO virus also improved the DNA transfection (Figure 4, no. 2) and indeed compensated for the 3-fold lower amount of pCLuc DNA when compared to the chloroquine experiment (Figure 4, no. 6). To determine the specificity of the DNA transfer, 1E3pL was replaced by free pL. In the case of human adenovirus, significant transgene expression was observed in the absence of 1E3pL (Figure 4, no. 3), suggesting its potential infectivity for human cells. In contrast, no CELO virus-mediated gene transfer was observed (Figure 4, no. 1), demonstrating the decreased binding capacity of human cells for the chicken virus. The use of 2- to 3-fold increased amounts of 1E3pL and DNA, keeping the ratio of 5:6 constant but without adenovirus, also improved transfection efficiency remarkably.

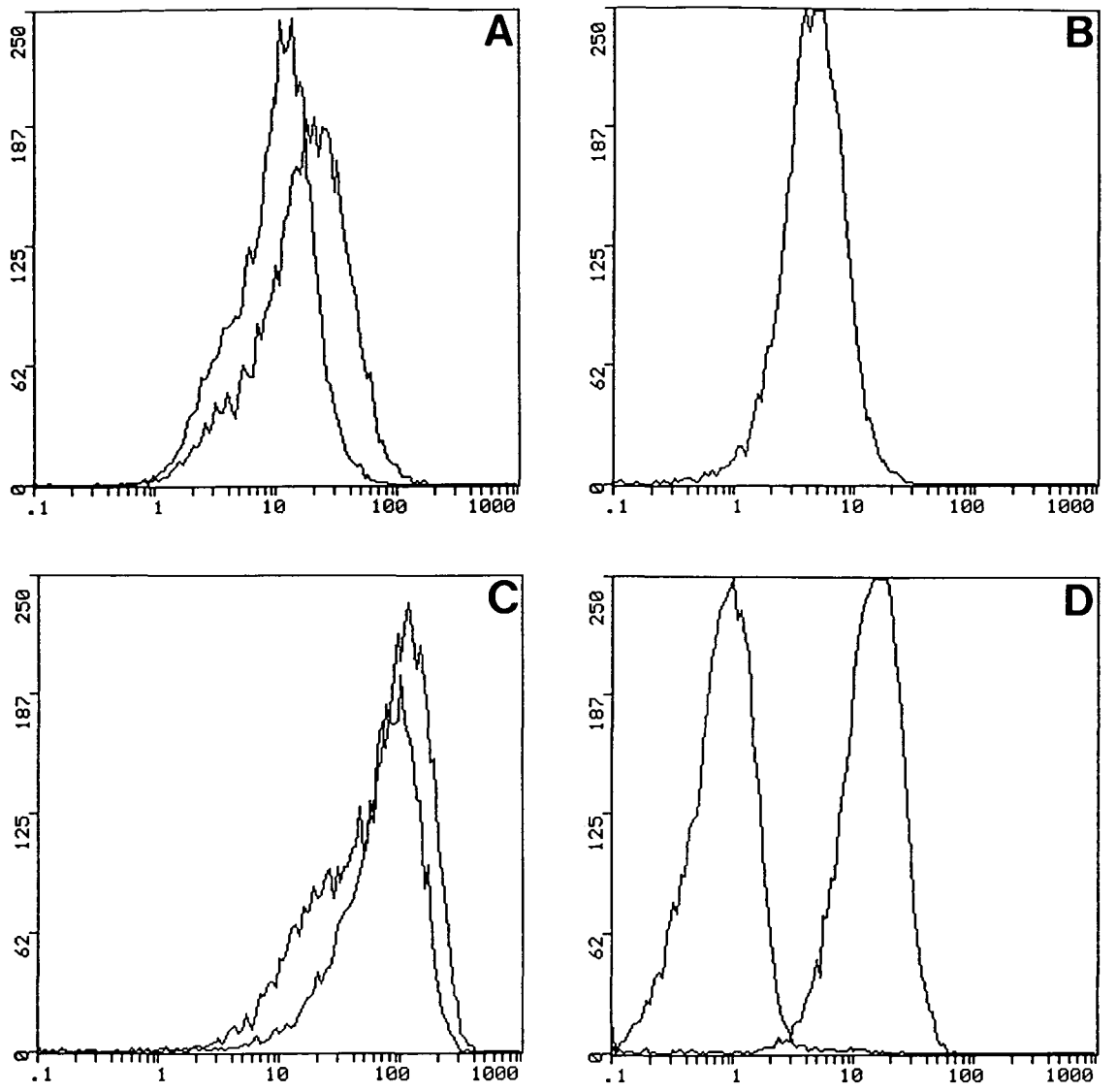

Fig. 1. Flow cytometric assessment of TfR, CD43 and Tn antigen expression. K562 (**A**) and Jurkat (**B**, **C**, **D**) cells were stained for flow cytometry as described in Materials and methods. Expression of TfR (mAb B3/25) was analysed on K562 cultivated in normal medium (left peak) or medium containing 50 μM desferrioxamine (A), and on Jurkat cells (B). Expression of Tn antigen (mAb 1E3) was analysed before (left peak) and after VCN treatment (C). CD43 expression on Jurkat cells is demonstrated by use of the sialidase-sensitive mAb DF-T1 (D). Antibody binding is abolished by VCN pre-treatment (left peak). The ordinate (number of cells) is linear, whereas the abscissa (cell fluorescence intensity) is a logarithmic scale. Mean fluorescence intensities (MFI) are presented in Table I.

Table I. Cell surface densities of TfR, CD43 and Tn antigen

Antigen	Synonym	Pre-treatment of cells	MFI	
			K562	Jurkat
TfR	CD71	–	9.537	4.546
		Desferrioxamine	16.580	n.d.
Leukosialin	CD43	–	n.d.	13.990
		VCN	n.d.	0.820
GalNAcα1-O-Ser/Thr	Tn antigen	–	n.d.	48.080
		VCN	n.d.	81.750

Table I summarizes the mean fluorescence intensities (MFI) of the flow cytograms presented in Figure 1. The MFI of the negative control (secondary antibody alone) was 0.524 (K562) and 0.209 (Jurkat), respectively. n.d. = not determined.

In this experiment, considerable luciferase activity was obtained even in the absence of chloroquine (Figure 4, no. 5), suggesting that the lysosomal degradation machinery or endosomal entrapment was being saturated and that DNA started to reach the nucleus under these conditions.

An extraordinary number of Jurkat cells express the transfected gene

To assess the percentage of cells expressing the delivered gene, we transfected the β-galactosidase (lacZ) gene under the control of the CMV promoter (MacGregor and Caskey, 1989) into Jurkat cells by Tn-mediated endocytosis. In the cytochemical staining presented in Figure 5, CELO virus samples resulted

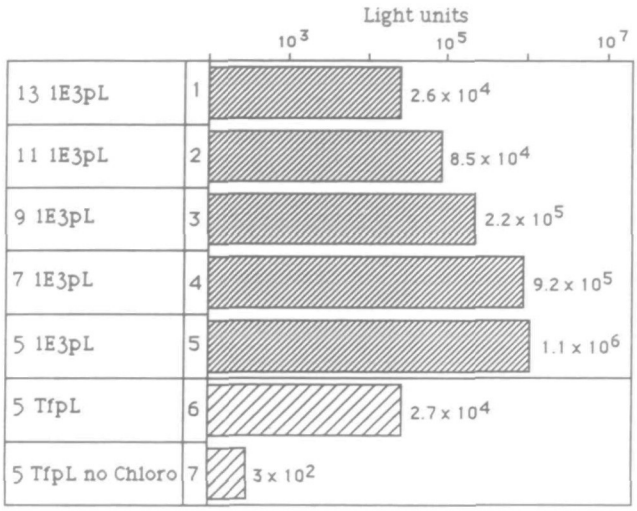

Fig. 2. Influence of the ratio of 1E3pL conjugate to pCLuc DNA on transfection efficiency. In a preliminary titration (nos 1–5), decreasing amounts of 1E3pL (from 13 to 5 μg) were mixed with a constant amount of pCLuc DNA (6 μg) and transfected into Jurkat cells in the presence of 100 μM of chloroquine. For comparison, a previously determined optimal ratio of TfpL and pCLuc DNA was delivered in the presence (no. 6) or absence (no. 7) of chloroquine. The indicated luciferase light unit values represent the averages of two transfections.

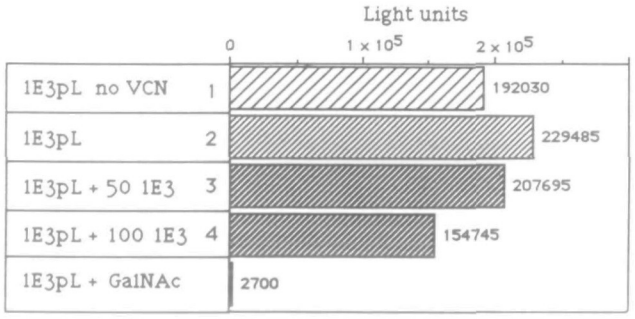

Fig. 3. Specificity of the DNA transfer using 1E3pL. Native (no. 1) or VCN-pre-treated Jurkat cells (no. 2) were transfected with the pre-determined optimal ratio of 1E3pL and DNA in the presence of chloroquine. As specificity controls, 50 μg (no. 3) or 100 μg (no. 4) of unconjugated 1E3 were added to the transfection medium (2.5 ml). In addition, the transfection was carried out in the presence of free GalNAc at 30 mM (no. 5). All transfections were set up in duplicate and mean values of luciferase activity are presented.

in β-galactosidase transgene expression in >60% of the cells. When human adenovirus was used, 26% stained for β-galactosidase activity. The relatively weak support of H5*dl*1014 is most likely due to an increased cellular toxicity as compared to CELO virus during a prolonged (48 h) expression time (Cotten *et al.*, 1993b). In experiments using chloroquine, 7% displayed β-galactosidase activity. In contrast, no blue cells were observed when pCLuc was transfected, indicating that transgene-encoded rather than endogenous galactosidase activity was responsible for blue colour development. Generally, T-lymphoid cells proved to be relatively resistant to high-efficiency transfection (Potter, 1988). With physical DNA transfer methods (as opposed to retroviral systems), transfection efficiencies of ~1% have been achieved so far (Bothwell, 1990). Thus, the

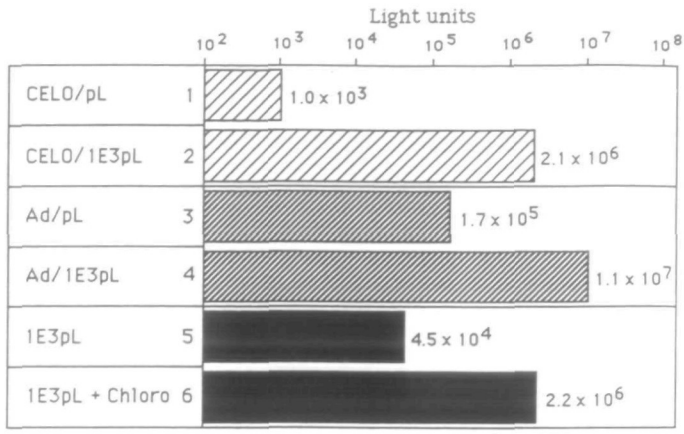

Fig. 4. Adenovirus particles augment DNA uptake. Human adenovirus (Ad) H5*dl*1014 or a chicken form of adenovirus (CELO) were used to augment Tn-mediated gene delivery. pL was attached to virus particles by a biotin–streptavidin linkage as described in Materials and methods. Biotinylated virus particles were assembled into complexes containing streptavidin–pL, DNA and, where indicated, 1E3 antibody–pL. To determine the specificity of the virus-augmented DNA transfer, 1E3pL was replaced by free pL (nos 1 and 3). While 5 μg of 1E3pL and 6 μg of pCLuc DNA were used for the virus-augmented DNA transfer (nos 2 and 4), 15 μg of 1E3pL and 18 μg of pCLuc DNA were also transfected in the absence (no. 5) or presence (no. 6) of chloroquine. The indicated luciferase light unit values are the averages of duplicate transfection experiments.

Tn-mediated gene transfer to Jurkat cells described here displays a considerably high efficiency, representing a major improvement of transfection efficiency over other physical methods.

Discussion

The first structure of a cell that a DNA-loaded ligand encounters is the glycocalyx, a dense coat of sugars surrounding each cell and serving various functions (Varki, 1993). It was, therefore, tempting to target a carbohydrate structure in gene transfer experiments. Cell surface carbohydrate antigens, and in particular mucin-like O-linked carbohydrate clusters, present several advantages as targets for the receptor-mediated gene delivery. One established role of these O-linked carbohydrates is that they confer a rod-like stem structure to the *O*-glycosylated protein which therefore extends from the cell surface (Carraway and Hull, 1991). At least physically, this protuberant structure makes surface mucins potential targets for gene delivery. Moreover, multiple O-linked carbohydrate side chains may be attached to a single protein molecule of the mucin type (Carraway and Hull, 1991). A major *O*-glycan-bearing glycoprotein of T lymphoid cells is leukosialin (= CD43) (Andersson and Gahmberg, 1978; Brown *et al.*, 1981). The number of TfRs expressed on K562 cells which are targeted in the transferrinfection method has been estimated to be $\sim 1.7 \times 10^5$/cell (Mattia *et al.*, 1984). Approximately 1.5×10^5 CD43 molecules are expressed per T cell (Barclay *et al.*, 1993). However, a single CD43 molecule may carry up to 80 *O*-glycosidic sugar chains (Fukuda, 1991), resulting in 1.2×10^7 CD43-associated Tn antigens on Jurkat cells. In addition, Tn antigens on Jurkat cells may also be attached to CD45, the leukocyte common antigen, which contains a mucin-like domain (Barclay *et al.*, 1993), indicating that the total number of Tn epitopes per

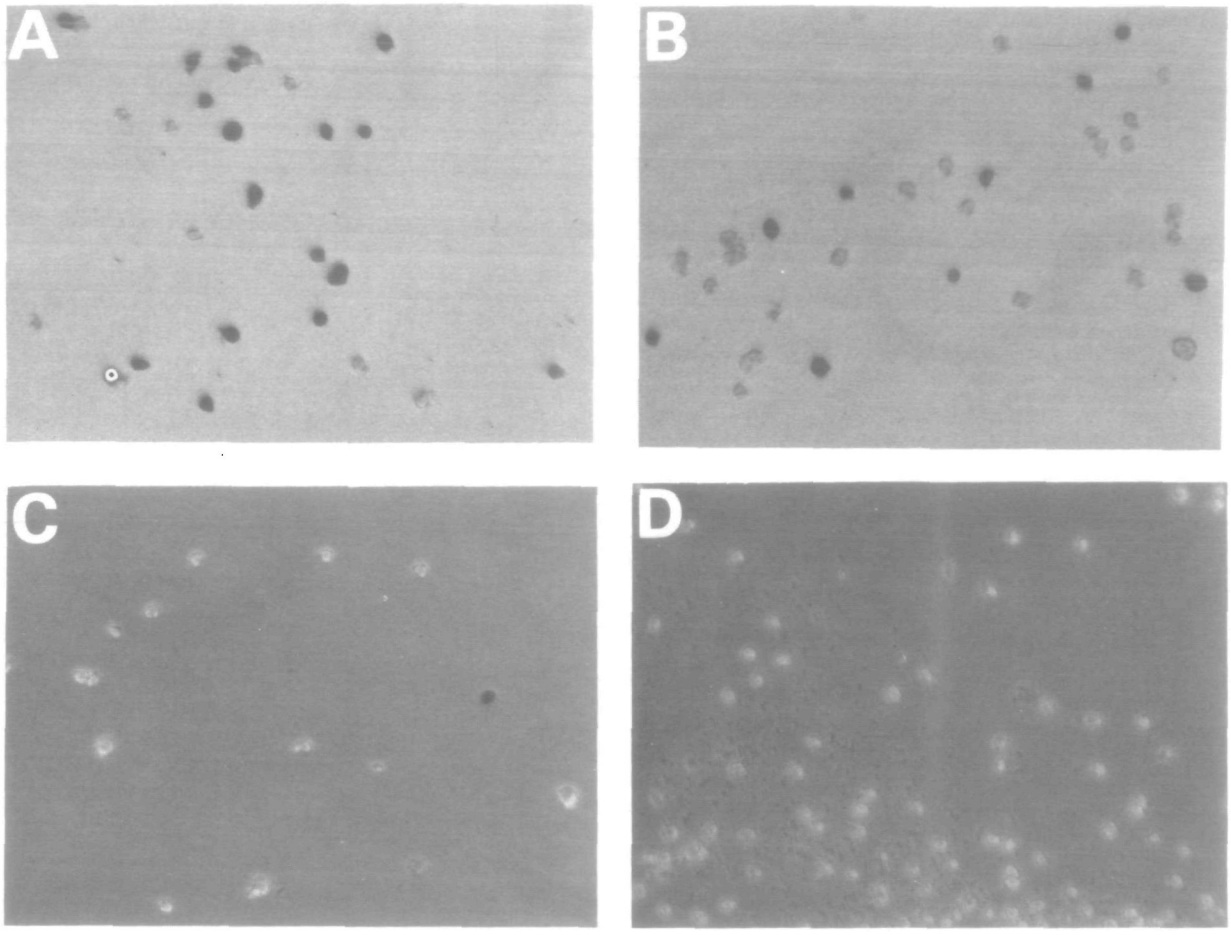

Fig. 5. Cytochemical staining of Tn-transfected cells. The *E.coli* β-galactosidase (lacZ) gene under the control of the CMV promoter was delivered to Jurkat cells by Tn-mediated endocytosis. β-Galactosidase activity was demonstrated *in situ* using X-gal as a chromogenic substrate. Gene transfer was augmented by CELO virus (**A**), H5*dl*1014 (**B**) or chloroquine (**C**). As a control for endogenous β-galactosidase activity, the pCLuc plasmid was delivered together with H5*dl*1014 (**D**). Magnification is ×100 and 150–350 cells were counted for each experiment.

Jurkat cell is even higher. Furthermore, Tn may be expressed on other Jurkat surface proteins which are not yet known to be *O*-glycosylated. Indeed, for approximately half of all clustered leukocyte antigens it is unknown whether they carry O-linked oligosaccharides or not (Barclay *et al.*, 1993). Taken together, it seems obvious that the cell surface density of carbohydrate receptors, in particular carbohydrate receptors of the mucin type, is much higher when compared to protein receptors. This calculation is also substantiated by the fluorescence intensities presented in Table I which indicate a 10- to 18-fold excess of Tn antigens over TfRs on Jurkat cells. It is reasonable to assume that this high carbohydate receptor density is mainly responsible for the efficient Tn-mediated gene delivery to Jurkat T cells described in Figures 2–5. However, it should be mentioned that the affinity and avidity of anti-carbohydrate antibodies is often lower as compared to anti-protein antibodies, even though no correlation has been done in this case. In addition, targeting mucin-type carbohydrate structures may not be relevant for all cells since, for example, mucin proteins in epithelial cells are preferentially secreted.

The carbohydrate receptor-mediated gene transfer described here will provide a useful tool for both basic T-cell research and for immunotherapy research which may require efficient transfection of tumour-infiltrating T lymphocytes (Rosenberg *et al.*, 1990). This approach may also be useful for the generation of genetically modified tumour cells as vaccines (Miller, 1992). Targeting of the Tn antigen and its derivative the sialosyl-Tn antigen, which are both expressed on many human primary carcinoma cells (Itzkowitz *et al.*, 1989; Springer, 1989), would allow the specific gene transfer to tumour cells, but not to normal cells also present in patient isolates. It is shown here that the gene transfer mediated by mucin-type carbohydrates may indeed be very efficient.

Materials and methods

Antibodies

mAb 1E3 was elicited using asialo ovine submaxillary mucin (asialo OSM) as immunogen which contains the Tn antigen as the major carbohydrate component (Schachter and Brockhausen, 1989). The hybridoma 1E3 secreting Ig2a was established using the NS-1 mouse myeloma fusion partner. 1E3 was readily inhibited by GalNAcα-*p*-nitrophenyl, but not by the β-anomeric configuration. In addition, we have previously shown that 1E3 selectively binds to Tn syndrome and Jurkat T cells (Thurnher *et al.*, 1992), both of which express the Tn antigen, but not to normal donor T cells which lack detectable Tn antigen. These data indicate that 1E3 indeed recognizes the Tn antigen. The anti-TfR mAb B3/25 was obtained from Boehringer. DF-T1, the sialic acid (NeuAc)-dependent (= sialidase-sensitive) anti-CD43 mAb was purchased from Dako. Binding of mAb was revealed by fluoresceinated goat anti-mouse Ig (Southern Biotechnology Assoc.).

Syntheses of conjugates

Human TfpL with an average chain length of 300 lysines were prepared as described previously (Wagner *et al.*, 1991a). mAb 1E3 was obtained from ascites after protein A purification and pL conjugates were prepared by conjugation through disulphide bridges after modification with *N*-succinimidyl 3-[2-pyridyldithio]propionate (SPDP) in analogous fashion as described previously (Wagner *et al.*, 1990). Conjugates were obtained containing 1E3 and pL (with an average chain length of 300 monomers) at a molar ratio of 1:2. Biotinylated adenoviruses (H5*dl*1014 or CELO) and streptavidin–pL conjugates were prepared as described previously (Wagner *et al.*, 1992).

Cell culture and transfection

The culture medium RPMI 1640 (Gibco) was supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% (v/v) heat-inactivated fetal calf serum (FCS) (Gibco). Jurkat cells were maintained at densities of 0.1–1 × 10^6 cells/ml. To ensure log-phase growth, cells were diluted the day before transfection. Transfections were performed in 24-well plates (2 ml/well) with 0.5 × 10^6 K562 cells or 1 × 10^6 Jurkat cells. To enhance TfR expression, K562 cells were pre-treated with 50 μM desferrioxamine for 18–24 h before transfection. When chloroquine was used, it was added just before the transfection to a final concentration of 100 μM. The plasmid pCLuc carrying the firefly luciferase gene is described by Plank *et al.* (1992), and the plasmid encoding the *Escherichia coli* β-galactosidase gene is described by MacGregor and Caskey (1989). DNA–1E3pL complexes were allowed to form in 500 μl of 150 mM NaCl and 20 mM HEPES (pH 7.4) (= HBS) within 30 min at room temperature and subsequently added to the cells. After a 4 h incubation at 37°C, chloroquine-containing medium was replaced by fresh culture medium and after an additional incubation for 24 (48) h, cells were harvested for luciferase (β-galactosidase) assay.

Human adenovirus H5*dl*1014 (Bridge and Ketner, 1989) was grown on W162 cells (Weinberg and Ketner, 1983) and purified by equilibrium banding in CsCl and biotinylated as described previously (Cotten *et al.*, 1993b). Complexes were allowed to assemble as described previously (Wagner *et al.*, 1992). Briefly, biotinylated adenoviruses (H5*dl*1014 or CELO, 10^{10} viral particles) were incubated with streptavidin–pL in 150 μl of HBS for 30 min at room temperature. Addition of excess pCLuc DNA in 150 μl of HBS resulted in incomplete condensation of the DNA. After another 30 min at room temperature, 1E3pL was added in 100 μl of HBS, causing full condensation of the DNA. After a further 30 min, the complexes consisting of virus, DNA and 1E3 antibody were added to the cells. After a 2 h incubation at 37°C, the transfection medium was replaced by fresh culture medium and after 24–48 h, as indicated, cells were again harvested for luciferase assay.

Reporter gene assays

Luciferase assay was carried out after 18–24 h of transgene expression according to Brasier and Ron (1992), and luciferase activity was determined in a luminometer (Berthold). The cytochemical staining for β-galactosidase using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) as a chromogenic substrate was performed after 48 h of expression according to Sanes *et al.* (1986) and cells were inspected using an Olympus IM inverted microscope equipped with an Olympus OM4 camera. Photographs were taken on Ektachrome 64T.

Sialidase treatment and flow cytometry

Samples of 10^7 Jurkat cells were desialylated with 20 mU VCN (Boehringer) in 1 ml phosphate-buffered saline (PBS) containing 0.5% (w/v) bovine serum albumin (BSA) for 1 h at 37°C. The cells were then washed twice with PBS containing 1% (v/v) FCS.

Cells were stained at 5 × 10^5 cells/100 μl sample for 30 min at 4°C. Cells were first stained with culture supernatant containing 1E3 (10 μg/ml) or with purified anti-TfR mAb (10 μg/ml) according to the manufacturer's instructions, followed by fluoresceinated goat anti-mouse Ig (50 μg/ml) in PBS containing 10% FCS. After each incubation, cells were washed twice with PBS containing 1% FCS. After the final wash, cells were fixed in 2% (v/v) formaldehyde in PBS containing 0.1% FCS (the fixative was filtered prior to use) and kept at 4°C in the dark until analysis. Cells were analysed for immunofluorescence on an EPICS Profile I flow cytometer. Data were presented with 'Elite' software from Coulter.

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Abbreviations

BSA, bovine serum albumin; CELO, chicken embryo lethal orphan; FCS, fetal calf serum; GalNAc, *N*-acetyl-D-galactosamine; β1,3Gal-T, β1,3 galactosyltransferase (EC 2.4.1.122); HIV, human immunodeficiency virus; MFI, mean fluorescence intensity; OSM, ovine submaxillary mucin; PBS, phosphate-buffered saline; pL, poly-L-lysine; SPDP, *N*-succinimidyl 3-[2-pyridyldithio] propionate; TfR, transferrin receptor; VCN, *Vibrio cholerae* sialidase; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

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