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Transferrin–polycation-mediated introduction of DNA into human leukemic cells: Stimulation by agents that affect the survival of transfected DNA or modulate transferrin receptor levels

(gene therapy/DNA transfection/endocytosis/chloroquine/desferrioxamine)

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ABSTRACT **We have subverted a receptor-mediated endocytosis event to transport genes into human leukemic cells. By coupling the natural iron-delivery protein transferrin to the DNA-binding polycations polylysine or protamine, we have created protein conjugates that bind nucleic acids and carry them into the cell during the normal transferrin cycle [Wagner, E., Zenke, M., Cotten, M., Beug, H. & Birnstiel, M. L. (1990) *Proc. Natl. Acad. Sci. USA* 87, 3410–3414]. We demonstrate here that this procedure is useful for a human leukemic cell line. We enhanced the rate of gene delivery by (*i*) increasing the transferrin receptor density through treatment of the cells with the cell-permeable iron chelator desferrioxamine, (*ii*) interfering with the synthesis of heme with succinyl acetone treatment, or (*iii*) stimulating the degradation of heme with cobalt chloride treatment. Consistent with gene delivery as an endocytosis event, we show that the subsequent expression in K-562 cells of a gene included in the transported DNA depends upon the cellular presence of the lysosomotropic agent chloroquine. By contrast, monensin blocks "*transferrin*fection," as does incubation of the cells at 18°C.**

Rapidly dividing cells and cells programmed to synthesize large quantities of heme—e.g., neoplastic cells or cells of the hematopoietic lineage—require elevated levels of iron. Cells obtain iron complexed with the carrier protein transferrin, which is then internalized after binding to a specific receptor (for review, see ref. 1). We have created human transferrin molecules that retain their ability to bind the transferrin receptor and are modified by the addition of a nucleic acid-binding domain (either protamine or polylysine) so that they can bind and carry nucleic acids into the cell. Complexes of polylysine–transferrin and DNA, when supplied to cells expressing the transferrin receptor, result in the uptake and expression of genes (2, 3) contained in the DNA. We call this DNA-transfer method "*transferrin*fection" (2). A similar strategy, employing the asialoglycoprotein receptor, has been used by Wu and Wu (4, 5) to deliver DNA to liver cells.

We have shown earlier that transferrin receptors play a pivotal role in transferrinfection of DNA and that the DNA is initially found in endosome-like particles (3). In view of the endosomal location of the DNA, degradation of at least some of the introduced nucleic acid would be anticipated as it reaches the lysosomal compartment. We have reported (2) that transferrinfection of DNA works efficiently for chicken erythroid cell lines and primary hematopoietic cells. Working to adapt the procedure for human cell lines, we sought to enhance the level of transferrinfected DNA in several different ways: (*i*) Chloroquine, a compound known to inhibit lysosomal hydrolytic enzymes such as proteases and nucleases (6), was included during transferrinfection. The purpose of using this drug was to try to increase the proportion of unscathed DNA reaching the nucleus and available for transcription. (*ii*) Cells were subjected to conditions that would be expected to increase transferrin-receptor levels (see legend for Fig. 1).

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MATERIALS AND METHODS

Preparation of Transferrinfection Complexes. Human polylysine–transferrin ($lysine_{270}$) conjugates were prepared and purified as described (2). The DNA plasmid pRSVL containing the *Photinus pyralis* luciferase gene under the control of the Rous sarcoma virus long terminal repeat enhancer/promoter (10) was diluted to 0.06 mg/ml in 150 mM NaCl/20 mM Hepes, pH 7.5. This DNA solution was added to a 2- to 3-fold transferrin mass excess of polycation–transferrin at a maximum concentration of 0.09 mg/ml (as transferrin) in 150 mM NaCl/20 mM Hepes, pH 7.5. Complexes were allowed to form for 30 min at room temperature before being added to cells.

Cells and Media. Cells of the human erythroleukemia cell line K-562 were grown in RPMI 1640 medium (supplemented with 10% fetal calf serum, penicillin at 100 international units/ml, streptomycin at 100 μg/ml, and 2 mM glutamine) at a density of 200,000–500,000 cells per ml. In some experiments cells were grown for 24–48 hr in the presence of test compounds, as indicated in the figure legends. In all cases, just before transferrinfection, the cells were washed twice with fresh medium and suspended at 150,000 per ml of RPMI 1640 medium containing, where indicated, 100 μM chloroquine. In some of the initial experiments, cells were washed with medium lacking serum, and the transferrinfections were performed in medium lacking serum. Test experiments showed that 10% fetal calf serum had no effect on transferrinfection efficiency (results not shown); subsequent experiments included 10% fetal calf serum. The DNA–polylysine–transferrin complex was added to the cell suspension for 4 hr at 37°C. At the end of this period, cells were transferred to fresh medium, either by centrifugation or by removing 90% of the cell medium, replacing it with fresh prewarmed medium, and repeating this maneuver after the cells resettled to the bottom of the dish. This procedure lowered chloroquine levels sufficiently to avoid long-term toxicity. The cells grew at 37°C for 18–48 hr, as indicated in each figure, after which they were harvested by centrifugation and washed twice with phosphate-buffered saline; extracts were then prepared, and aliquots, standardized for protein content, were analyzed for luciferase activity (see ref. 2).

RESULTS

Requirement for Chloroquine During the Transferrinfection Period. We have tested the requirement of the human leukemic cell line K-562 for chloroquine during the 4-hr transferrinfection period. Luciferase activity was assayed in cells

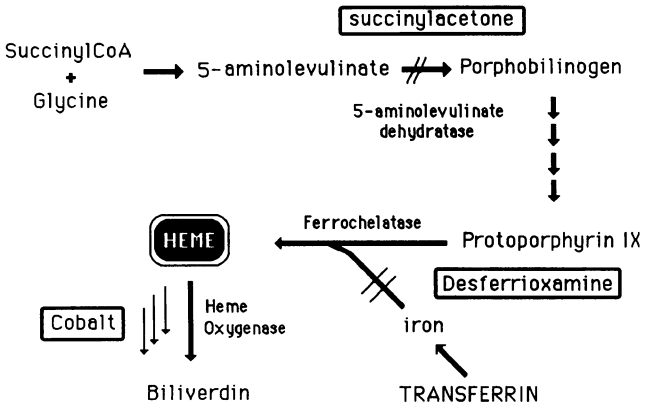

FIG. 1. Schematic representation of heme metabolism. Compounds that alter the internal iron levels or modify the level of hemin, either by decreasing heme synthesis or by increasing heme degradation, modulate transferrin-receptor levels (7). When one treats the cells with the cell-permeable iron chelator desferrioxamine, the cell responds to the iron deficit by increasing the number of transferrin receptors on its surface (7, 8, 25). Succinyl acetone is a potent inhibitor of the first committed step of heme biosynthesis, 5-aminolevulinate acid dehydratase (7); treatment of cells with succinyl acetone increases cell-surface transferrin receptors (7, 25). When cobalt chloride is added to cells, heme oxygenase and, hence, heme degradation is stimulated, and the cell responds by increasing transferrin-receptor levels (7).

transferrinfected with either 3 or 10 μg of DNA (complexed with a 3-fold mass excess of transferrin–polylysine conjugate) for 4 hr either with or without 100 μM chloroquine. We found no activity in the absence of chloroquine (Fig. 2). However, with chloroquine luciferase activity was obtained—greater quantities of luciferase activity being obtained with higher quantities of DNA per polylysine–transferrin.

The carboxylic acid ionophore monensin acts on the Golgi apparatus and is also a potent lysosomotropic agent (11). It has been reported to interfere with the transferrin cycle by blocking vesicle fusion and the return of the apotransferrin–transferrin receptor complex to the cell surface (14). We tested the transferrinfection with monensin to determine whether this agent would behave in a manner similar to chloroquine. Monensin, at low concentration (1–10 μM), was ineffective in stimulating transferrinfection (Fig. 3) and actually interfered with the delivery of DNA. When cells were treated with both 100 μM chloroquine (which alone gave a strong luciferase signal) and monensin, the resulting luciferase activity was negligible (Fig. 3).

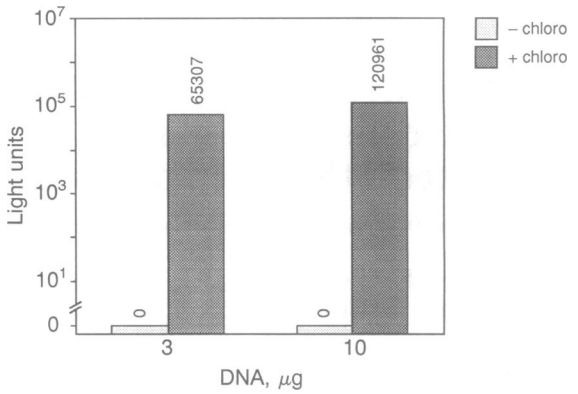

FIG. 2. Influence of chloroquine on transferrinfection. Samples of K-562 cells (300,000 cells per assay point) were transferrinfected for 4 hr with either 3 or 10 μg of pRSVL with or without 100 μM chloroquine (chloro). Extracts were made, and luciferase activity was measured 48 hr later in aliquots standardized for protein content. The activity shown is adjusted to represent total activity from the entire cellular sample.

Effects of Desferrioxamine Pretreatment. K-562 cells were grown for 20 hr with 50 μM desferrioxamine in an attempt to elevate the level of transferrin receptor and, in turn, to increase the amount of DNA delivered to cells. Both control cells and desferrioxamine-treated cells expressed increased luciferase activity with increased DNA supplied to the cells as a polylysine–transferrin conjugate (1, 3, and 10 μg of DNA, Fig. 4). We found that a desferrioxamine pretreatment resulted in a 4-fold increase in transferrin receptor number (M.C., unpublished results) and in a 5-fold increase in luciferase activity over control cells with 3 μg of DNA and a 15-fold increase with 10 μg of DNA.

Effects of Desferrioxamine During the Transferrinfection Period. In the above experiments desferrioxamine was present during the transfection period. There was some concern that this compound might remove iron from the transferrin–polylysine conjugates—thus blocking binding of the conjugates to the transferrin receptors on the cell surface. The effect of desferrioxamine during the transfection period was therefore tested directly. Cells, with or without a 48-hr pretreatment of 50 μM desferrioxamine, were transferrinfected for 4 hr in the presence of 100 μM chloroquine with or without 50 μM desferrioxamine. We found that in all cases (with or without desferrioxamine pretreatment) desferrioxamine during the transferrinfection period enhanced subsequent luciferase activity (Fig. 5). Even without desferriox-

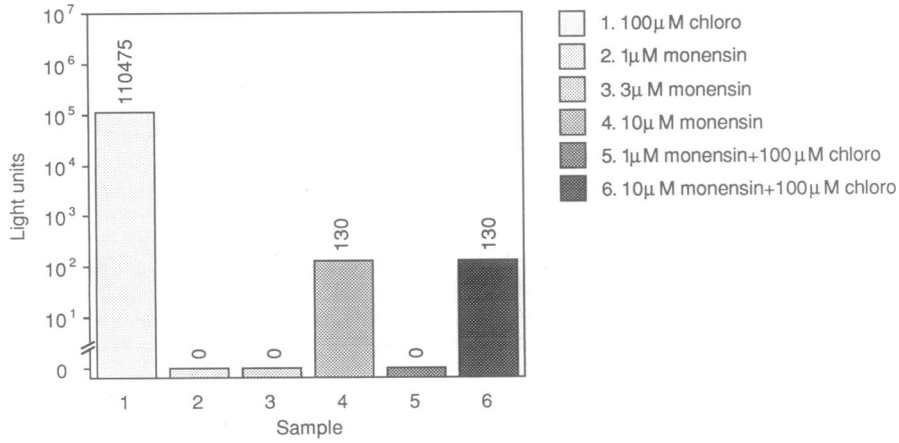

FIG. 3. Influence of monensin on transferrinfection. Transferrinfection was performed as for Fig. 2. During the transferrinfection period samples included 100 μM chloroquine (chloro); 1, 3, 10, or 30 μM monensin; or 1 or 10 μM monensin plus 100 μM chloroquine.

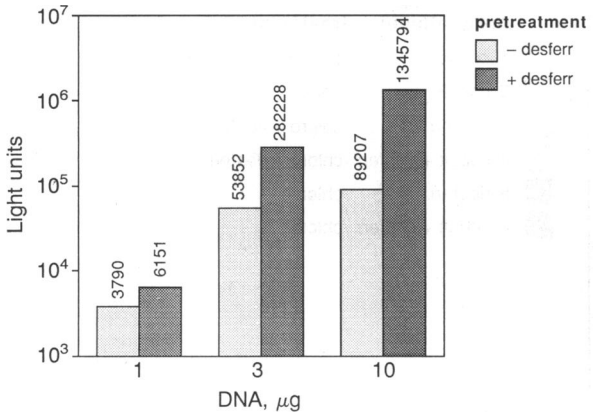

FIG. 4. Influence of desferrioxamine on transferrinfection. K-562 cells were grown with 50 μM desferrioxamine (desferr) for 24 hr. Samples of either control or desferrioxamine-treated cells (300,000 cells per assay point) were transferrinfected for 4 hr with 1, 3, or 10 μg of pRSVL in the presence of 100 μM chloroquine. Desferrioxamine-treated cells also contained 50 μM desferrioxamine during the transfection period. Cell extracts were made 48 hr after infection, and luciferase activity was determined as for Fig. 2.

amine pretreatment, the presence of desferrioxamine produces a 10-fold stimulation of expression. Chloroquine was required for the desferrioxamine effect. Note that in our standard procedure the transferrin–polylysine complexes contain a 50-fold molar excess of iron citrate (over transferrin) to ensure complete iron loading of the transferrin. We suspect that the cells may well respond to this iron excess by promptly downregulating the transferrin receptor cycling. The desferrioxamine present during the transferrinfection may serve to chelate excess iron and to maintain high transferrin receptor levels; desferrioxamine may also influence endosomal "sorting" in subtle ways (see *Discussion*).

The high expression levels with desferrioxamine pretreatment depended on the presence of chloroquine during the 4-hr transferrinfection period; expression fell to background levels without chloroquine (Fig. 5). Raising the transferrin receptor density is apparently not sufficient in itself to elevate transferrinfection levels. Some additional event, modulated by chloroquine, limits the DNA expression.

Effects of Cobalt Chloride Pretreatment. Cobalt chloride is reported (7) to stimulate heme oxygenase activity, which results in an increase in transferrin-binding activity in HeLa cells. We tested the hypothesis that cobalt pretreatment also

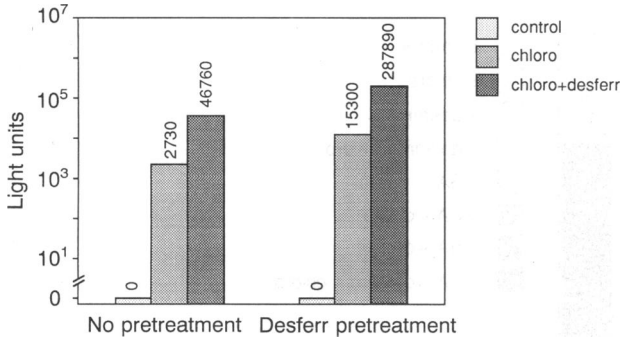

FIG. 5. Influence of desferrioxamine (desferr) and chloroquine (chloro) on transferrinfection. K-562 cells were grown in the presence of 50 μM desferrioxamine for 24 hr. Samples of either control or desferrioxamine-treated cells (300,000 cells per assay point) were transferrinfected for 4 hr with 10 μg of pRSVL in the absence or presence of 100 μM chloroquine and 50 μM desferrioxamine, as indicated. Cell extracts were made 48 hr after infection, and luciferase activity was determined as for Fig. 2.

increases expression of a transferrinfected luciferase gene similar to that obtained with desferrioxamine. Cells were exposed to either 50 μM desferrioxamine or 130 μM cobalt chloride or both compounds for 48 hr before transferrinfection. In samples pretreated with desferrioxamine, the compound was also present during the transferrinfection period. Both 130 μM cobalt chloride and 50 μM desferrioxamine yielded similar enhancements of luciferase expression; combination of the two compounds resulted in an additive increase of expression (Fig. 6). The additive effect of desferrioxamine and cobalt was also seen when desferrioxamine was omitted from the sample during the transfection period. Although the level of expression was lower without desferrioxamine during transferrinfection, the cobalt plus desferrioxamine-pretreated sample still showed twice the level of expression relative to the desferrioxamine-pretreated sample.

Effects of Succinyl Acetone Pretreatment. Succinyl acetone inhibits 5-aminolevulinic acid dehydratase, the major controlling enzyme for heme biosynthesis. Treatment of cells with succinyl acetone has been reported (7, 25) to upregulate transferrin receptor levels. To test the effect of this compound on transferrinfection, K-562 cells were incubated in 50 μM desferrioxamine, 2 mM succinyl acetone, or a combination of both compounds for 48 hr. Transferrinfection was performed for 4 hr in the continued presence of these compounds with or without 100 μM chloroquine. The cells were harvested 18 hr later and assayed for luciferase activity. Succinyl acetone pretreatment modestly increased luciferase activity compared with untreated cells, and this activity depended on the presence of chloroquine during the transferrinfection period (Fig. 7). The succinyl acetone stimulation was not as great as the desferrioxamine or cobalt stimulations; however, the combination of desferrioxamine and succinyl acetone provided a slight, but reproducible, stimulation over either compound alone.

DISCUSSION

Our initial goal was to develop methods for the transport of antisense oligonucleotides and ribozymes across the cell membrane. We soon discovered that the same modified receptor-mediated-transport system transferred high-molecular-weight plasmids containing the luciferase gene with relatively high efficiency. This observation allowed us to rapidly and reliably monitor the introduction of nucleic acids into the cell and their transfer to the nucleus. Therefore, we decided to optimize the transferrinfection procedure by using this simple reporter system for later application of the technique to antisense oligonucleotides and ribozymes.

In this paper optimal conditions for the introduction of DNA into K-562 cells are established using a subverted cellular receptor-mediated endocytosis mechanism, referred to as transferrinfection (2). The K-562 cells used for our studies exhibit the response anticipated toward drugs known to modulate the intracellular level of iron-heme (Fig. 1) and, consequently, transferrin-receptor levels. Reduction of intracellular iron-heme concentration either by inhibiting formation of porphobilinogen, the heme precursor, with succinyl acetone or by complexing iron ions with desferrioxamine upregulates transferrin receptors (7–9), and we observed that under these conditions cells become more receptive to transferrinfection. Similarly, we have shown that treatment of cells with cobalt chloride (probably resulting in increased heme oxygenase degradation of heme) increases the import of DNA during transferrinfection of plasmids. In all these cases combinations of treatment show additive effects, as measured by luciferase gene expression.

In K-562 cells expression of an imported luciferase gene depends on the presence of chloroquine during transferrin-

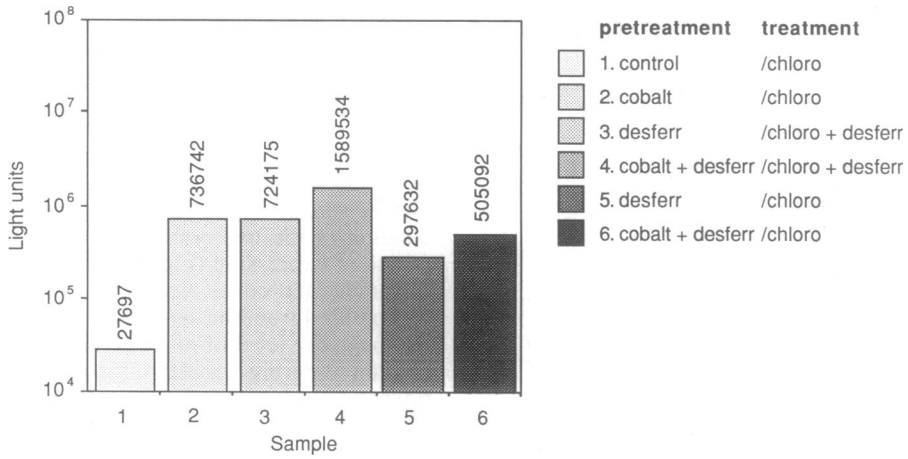

FIG. 6. Influence of cobalt chloride on transferrinfection. K-562 cells were grown in the presence of 130 μM cobalt chloride, 50 μM desferrioxamine (desferr), or a combination of both compounds, for 48 hr. Samples of either control or desferrioxamine-treated cells (300,000 cells per assay point) were transferrinfected for 4 hr with 10 μg of pRSVL in the absence or presence of 100 μM chloroquine (chloro) and/or 50 μM desferrioxamine, as indicated. Cell extracts were made 48 hr after infection, and luciferase activity was determined as for Fig. 2.

fection. We presume that chloroquine inhibits lysosomal nucleases to a sufficient degree to allow survival, nuclear import, and expression of at least some of the transfected pRSVL plasmid molecules. However, a second lysosomotropic agent, monensin, does not facilitate transferrinfection and actually interferes with it.

We have tested chloroquine at different concentrations and find that at 50 μM or less chloroquine is nontoxic to cells but is also ineffective. Chloroquine at the high concentrations necessary to augment luciferase expression (100 μM) is toxic to the cells upon protracted incubation. Cells exposed to the drug at 100 μM for >12 hr die progressively with incubation time, although cells appear to tolerate incubation of 4 hr without deleterious effects. The necessity for chloroquine inclusion during transfection seen for K-562 cells is by no means a universal feature of transferrinfection because cell lines differ considerably with respect to this requirement. Thus, an erythroblastic chicken cell line shows high levels of transferrin-mediated DNA importation, even without chloroquine (3). We do not yet understand why cells differ so drastically in their response to chloroquine during transferrinfection.

As judged from our work with inhibitors, transferrin and transferrin conjugates appear to follow different pathways in the vesicular system of the cell. Time-course experiments by others reveal initial localization of the unmodified transferrin and receptor in endocytic vesicles near the cell surface of K-562 cells with subsequent localization in multivesicular bodies in the peri-Golgi region of the cell (12), followed by rapid exocytosis (13). The transferrin-receptor system differs from many other cellular ligands and their receptors in that the transferrin cycle does not interact with the lysosomal compartment. As pH is lowered in the endosome, Fe^{3+} ions are set free and the apotransferrin and receptor remain complexed, whereas other ligands and receptors dissociate under these conditions. The "sorting endosome" (13) elicits transport of the transferrin receptors by means of the "recycling endosome" to the cell surface—still associated with transferrin. Other types of ligands are set free with the sorting endosome at the prevailing low pH, are held back, and enter "late" endosomes and finally the lysosomal compartment. Dissociation of the apotransferrin from its receptor occurs at high pH after the receptors have surfaced from the cell (1). Monensin, but not chloroquine, apparently stops transferrin recycling (14) within the peri-Golgi complex, as does chilling cells to 18°C (13), and prevents the lysosomal routing of ligands (other than transferrin), but, like chloroquine, additionally exerts effects through alkalinization of the lysosomal content and/or prelysosomal compartments (11). The behavior of the DNA from the transferrin–polylysine–DNA complex differs from that of the nonconjugated transferrin-receptor complex in that the DNA probably enters the

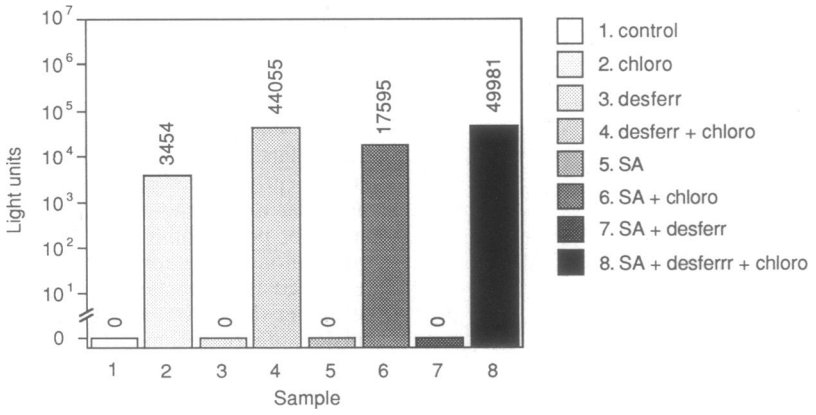

FIG. 7. Influence of succinyl acetone on transferrinfection. K-562 cells were grown in the presence of 2 mM succinyl acetone (SA), 50 μM desferrioxamine (desferr), or a combination of both compounds for 48 hr. Samples of either control or desferrioxamine-treated cells (300,000 cells per assay point) were transferrinfected for 4 hr with 10 μg of pRSVL in the absence or presence of 100 μM chloroquine (chloro) and/or 50 μM desferrioxamine, as indicated. Cell extracts were made 48 hr after infection, and luciferase activity was determined as for Fig. 2.

lysosomal complex, as suggested by the complete inhibition of transferrinfection by monensin (Fig. 3) or by chilling cells to 18°C (data not shown) and the strong stimulation of transferrinfection by chloroquine (Fig. 2). Thus, transferrin–polylysine–DNA complexes may behave like transferrin-colloidal gold or transferrin crosslinked with anti-transferrin antibodies, which are known to become directed to lysosomes (15, 16).

Why are we working to develop a new transfection technique when so many methods are already available? The simplest answer is that the available techniques are far from adequate. The major techniques being considered for gene therapy use—namely, retroviral vectors, electroporation, $CaPO_4$ precipitation, and microinjection—have numerous drawbacks. Microinjection is time consuming, and each individual cell must be manipulated, which is a difficult or even impossible task for most applications. Retroviral vectors have limited gene capacity and can activate oncogenes upon insertion in the target chromosome. Electroporation and $CaPO_4$ precipitation are harsh, unphysiological, and cause much cell death.

Is transferrinfection any better? Certainly the delivery capacity, at least in K-562 cells, is as high as any of the available techniques. We find that transferrinfection of K-562 cells results in 10- to 30-fold higher expression of luciferase than that obtained with a DEAE-dextran protocol (17). Transferrinfection can be repeated several times on the same cell culture to increase the level of DNA transfer without causing cell death (3). Transferrinfection is applicable to primary cell cultures (3), but we caution that cells differ drastically in their response to transferrinfection. Where the technique works there is no overt limit to DNA size, and thus far DNA molecules as large as 14,000 base pairs have been successfully transferred. This is an extra bonus because eukaryotic genes can be very large. The requirement for chloroquine for maximal transferrinfection into certain cells could be a drawback because this requirement might limit application of the technique in living organisms; however, this disadvantage may not prevent its use in *ex vivo* treatment procedures. Desferrioxamine, on the other hand, is a drug tolerated at high plasma concentration, which could possibly be used to increase receptor levels *in vivo*.

One general advantage of receptor-mediated endocytosis of DNA is the ability to target DNA to specific cells by coupling the DNA-binding domain to cell-specific ligands and thus generating cell-specific DNA-delivery conjugates. As mentioned above, high levels of transferrin receptors are seen in proliferating and/or neoplastic cells (1, 18, 19). We note, for instance, that normal breast tissue or cervical epithelium, when challenged with transferrin-receptor antibodies, exhibits no significant transferrin-receptor binding, whereas a majority of breast (16 out of 22) and almost all uterine cervical carcinomas (33 out of 34) show extensive reactivity with anti-transferrin-receptor antibodies (20–23). Transferrin-receptor expression in leukemia/lymphoma cells seems to reflect their proliferative status (ref. 24 and references therein). It will be worthwhile to test whether transferrinfection can be extended to cells other than K-562 exhibiting high levels of transferrin receptor expression.

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