0026-895X/92/061023-11$03.00/0

MOLECULAR PHARMACOLOGY, 41:1023-1033

Cationic Lipids Enhance Cellular Uptake and Activity of Phosphorothioate Antisense Oligonucleotides

C. FRANK BENNETT, MING-YI CHIANG, HEDY CHAN, JO ELLEN E. SHOEMAKER, and CHRISTOPHER K. MIRABELLI
Department of Molecular and Cellular Biology, ISIS Pharmaceuticals, Carlsbad, California 92008
Received October 23, 1991; Accepted February 27, 1992

SUMMARY

We have investigated the use of a cationic lipid preparation to enhance antisense oligonucleotide activity in human umbilical vein endothelial cells. A liposomal preparation containing the cationic lipid *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) was found to increase by at least 1000-fold the potency of an antisense oligonucleotide (ISIS 1570) that hybridizes to the AUG translation initiation codon of human intercellular adhesion molecule-1. In the presence of 8 μM DOTMA, 6–15-fold more ^{35}S-ISIS 1570 associated with cells, at oligonucleotide concentrations from 0.01 to 5 μM, than did in the absence of DOTMA. Both ^{35}S-ISIS 1570 association with cells and antisense activity were increased as a function of DOTMA concentration and with increasing time of incubation with the cationic lipid. Fluorescein-labeled ISIS 1570 was used to assess the intracellular distribution of the oligonucleotide in the presence and absence of DOTMA. In the absence of DOTMA, the oligonucleotide localized to discrete structures in the cytoplasm of the cell, resulting in a punctate fluorescence pattern. In the presence of DOTMA, cellular fluorescence markedly increased and the oligonucleotide localized within the nucleus, as well as to discrete structures in the cytoplasm. Accumulation of the oligonucleotide in the nucleus in the presence of DOTMA was time and temperature dependent. Nuclear accumulation was inhibited by preincubation of the cells with monensin but not chloroquine, NH_4Cl, nocodazole, colcemid, or brefeldin A. These data demonstrate that cationic lipids increase antisense activity by increasing the amount of oligonucleotide associated with cells and altering intracellular distribution of the oligonucleotide.

Oligonucleotides designed to hybridize to specific mRNA sequences have been used to inhibit the function of a number of cellular and viral proteins (reviewed in Refs. 1–4). These antisense oligonucleotides bind to mRNA or pre-mRNA through normal Watson-Crick base pairing. Thus, if the DNA sequence of a protein of interest is known, it is possible to design complementary oligonucleotides that bind the target RNA and inhibit expression of the protein. Unmodified oligodeoxyribonucleotides have been used to inhibit the expression of several viral and cellular encoded proteins in cell culture (1–13). However, these compounds are unstable in biological fluids and display poor cellular uptake characteristics (1–4). Numerous modifications of the phosphodiester backbone have been performed to decrease the sensitivity of the oligonucleotides to nucleases and/or to increase cellular uptake, yielding, for example, phosphotriesters, methylphosphonates, phosphorothioates, phosphorodithioate, phosphoroselenoate, and α-anomers (1–4).

Due in part to readily available commercial supplies of monomers and facile automated synthesis, methylphosphonates and phosphorothioates are the most widely used modified oligonucleotides in cellular assays. Both types of oligonucleotides exhibit enhanced nuclease resistance. Elimination of the negative charge on the phosphate backbone in the methylphosphonates has been reported also to enhance cellular uptake (1, 2, 4, 14, 15). However, these compounds also exhibit decreased solubility in aqueous solutions. Phosphorothioate oligonucleotides retain the negative charge on the phosphorous backbone and, like unmodified deoxyoligonucleotides, are substrates for RNase H, which may function as a terminating event in mammalian cells (1, 2, 4, 16). In contrast, methylphosphonate-modified oligonucleotides are not substrates for RNase H (1, 2, 4, 16).

ICAM-1 is a 90–110-kDa membrane glycoprotein involved in the trafficking of leukocytes out of the vasculature and in antigen presentation to T cells (17, 18). ICAM-1 is normally expressed at low levels on the surface of endothelial cells, keratinocytes, fibroblasts, and leukocytes. Expression of ICAM-1 is inducible by a number of cytokines, including IL-1β, tumor necrosis factor-α, and interferon-γ (17–20). Increased expression of ICAM-1 has been demonstrated in a variety of human diseases and has been shown to correlate with leukocyte infiltration in the diseased tissue (21–24). Inhibition of ICAM-1 expression is predicted to attenuate leukocyte infiltration into inflamed tissue, resulting in anti-inflammatory activity.

ABBREVIATIONS: ICAM-1, intracellular adhesion molecule-1; DOTMA, *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride; D-PBS, Dulbecco's phosphate-buffered saline; EGM-UV, endothelial cell growth medium-umbilical vein; FITC, fluorescein isothiocyanate; HUVEC, human umbilical vein endothelial cells; IL-1β, interleukin 1β; RITC, rhodamine isothiocyanate; ELISA, enzyme-linked immunosorbent assay.

Previously, we demonstrated that, in the presence of cationic lipids, specific phosphorothioate antisense oligonucleotides were capable of selectively inhibiting ICAM-1 expression (25). The antisense oligonucleotide inhibited ICAM-1 expression by at least two mechanisms, one of which resulted in a loss of the target mRNA (25). Subsequently, using antisense oligonucleotides designed to hybridize to ICAM-1, E-selectin, and vascular cell adhesion molecule-1 mRNAs, we have been able to demonstrate selective inhibition of the respective protein without affecting the synthesis of the other endothelial cell adhesion molecule, demonstrating the specificity of the antisense oligonucleotides.[1] During the course of our studies on inhibition of ICAM-1 expression with antisense oligonucleotides, we observed very modest inhibitory activity with phosphorothioate-containing oligonucleotides and no inhibitory activity with unmodified oligonucleotides. In an attempt to enhance cellular uptake of the oligonucleotides, we found that cationic lipids such as DOTMA (26) greatly increased biological activity of phosphorothioate-containing oligonucleotides. We now report the results of our studies on the mechanisms by which DOTMA enhances biological activity of phosphorothioate antisense oligonucleotides.

Materials and Methods

Cells and reagents. HUVEC were obtained from Clonetics (San Diego, CA) and cultivated in EGM-UV (Clonetics). HUVEC were used between the second and sixth passages. Human recombinant IL-1β and tumor necrosis factor-α were purchased from R&D Systems (Minneapolis, MN). Human fibronectin and chlorophenol red-β-D-galactopyranoside were purchased from Boehringer Mannheim (Indianapolis, IN). ICAM-1 monoclonal antibody 84H10 was purchased from AMAC Inc. (Westbrook, ME). DOTMA solution (Lipofectin), biotinylated goat anti-mouse, and β-galactosidase-conjugated streptavidin were purchased from Bethesda Research Laboratories (Bethesda, MD). Controlled pore glass-bound DNA synthesizer reagents and β-cyanoethyldiisopropylphosphoramidites were purchased from Applied Biosystems (Foster City, CA). Opti-MEM was purchased from GIBCO (Grand Island, NY). Fetal calf serum and D-PBS were purchased from Irvine Scientific (Irvine, CA). Centrex filters were purchased from Schleicher and Schuell (Keene, NH). $^{35}S_8$ (octaatomic sulfur) was purchased from Amersham (Arlington Heights, IL). FITC and RITC were purchased from Sigma Chemical Co. (St. Louis, MO). Electron microscopy-grade formaldehyde was purchased from Polysciences (Warrington, PA). Primaria 96-well and 24-well plates were purchased from Falcon (Lincoln Park, NJ).

Oligonucleotide synthesis. Unmodified DNA oligonucleotides were synthesized on an Applied Biosystems model 380B automated DNA synthesizer (Foster City, CA), using standard phosphoramidate chemistry with oxidation by iodine. For phosphorothioate oligodeoxyribonucleotides, the standard oxidation bottle was replaced with a 0.2 M solution of 3H-1,2-benzodithiole-3-one 1,1-dioxide (27) in acetonitrile, for stepwise sulfurization of the phosphite linkages. The sulfurization wait step was increased to 68 sec and was followed by the capping step. After cleavage from the controlled pore glass column and deblocking in concentrated ammonium hydroxide at 55° for 18 hr, the oligonucleotides were purified by precipitation, two times, out of 0.5 M NaCl solution with ethanol. Analytical gel electrophoresis was accomplished in 20% acrylamide, 8 M urea, 45 mM Tris-borate buffer, pH 7.0. Oligodeoxynucleotides and their phosphorothioate analogs were judged from polyacrylamide gel electrophoresis to be >80% full length material.

[1] C. F. Bennett *et al.* Manuscript in preparation.

Oligonucleotide labeling. Oligonucleotides were labeled with ^{35}S at each internucleosidic bond, as described by Stein *et al.* (28). ISIS 1570 (5′-TGGGAGCCATAGCGAGGC-3′) was synthesized on 1-μmol columns, using hydrogen phosphate chemistry, and was sulfurized with 1 mCi/μmol $^{35}S_8$, as described previously (28). Final specific activity of the labeled oligonucleotide was 7×10^7 cpm/μmol. Oligonucleotides were determined to be >80% full length material, by polyacrylamide gel electrophoresis followed by autoradiography. For labeling with fluorescent reporter groups, oligonucleotides were synthesized with a six-carbon spacer attached to a 3′-amine group (Applied Biosystem Inc.), using standard phosphoramidite chemistry as described above. Oligonucleotides were labeled with fluorescein or rhodamine by incubating 0.1 μmol of oligonucleotide with 0.6 μmol of FITC or RITC in 1 ml of 0.1 M sodium carbonate, pH 9.0, for 18 hr at 25°. The reaction was stopped by the addition of 50 μl of 100 mM NH_4Cl and was incubated for 2 hr at 25°. Oligonucleotide was separated from free FITC or RITC by gel filtration, using Sephadex G-25 equilibrated with deionized water. Oligonucleotides were lyophilized dry and further purified by precipitation with ethanol twice. Contamination with free FITC or RITC was <0.5%, as determined by reverse phase high performance liquid chromatography.

Oligonucleotide treatment of cells. Cells were washed three times with Opti-MEM prewarmed to 37°. Opti-MEM containing indicated concentrations of DOTMA solution was added to each well of the plate (100 μl). Oligonucleotides were sterilized by centrifugation through 0.2-μm Centrex cellulose acetate filters. Oligonucleotides were added to the wells as 20× stock solutions and were incubated for 4 hr at 37°. Medium was removed and replaced with the appropriate cell growth medium (150 μl) containing the indicated concentration of oligonucleotide. Cells were incubated for an additional 3–4 hr at 37° and then stimulated with the appropriate cytokine for 14–16 hr, as indicated. ICAM-1 expression was determined as described below.

ICAM-1 assay. ICAM-1 expression on the cell surface was determined by ELISA, using cells grown to confluence in 96-well plates. Cells were washed three times with D-PBS and fixed for 20 min in 2% formaldehyde diluted in D-PBS. The cells were washed three times with D-PBS, blocked for 1 hr at 37° with 2% bovine serum albumin in D-PBS, and incubated with the ICAM-1 monoclonal antibody 84H10 (0.5 μg/ml) for 1 hr at 37°. Antibody bound to the cells was determined by incubation with a 1/1000 dilution of biotinylated goat anti-mouse IgG followed by incubation with a 1/1000 dilution of β-galactosidase-conjugated streptavidin. The plates were developed with 100 μl of 3.3 mM chlorophenol red-β-D-galactopyranoside in 50 mM sodium phosphate, 1.5 mM $MgCl_2$, pH 7.0. Product formed was detected by the absorbance at 575 nm. The data were expressed as percentage of control activity, which was calculated as follows: [((ICAM-1 expression for oligonucleotide-treated cytokine-induced cells) − (basal ICAM-1 expression))/((cytokine-induced ICAM-1 expression) − (basal ICAM-1 expression))] × 100. Both basal and cytokine-treated cells were pretreated with DOTMA.

Cell association of ^{35}S-ISIS 1570. Cells grown in 24-well plates were washed three times with Opti-MEM prewarmed to 37°. DOTMA solution and oligonucleotide were added to the cells at the indicated concentrations. At the indicated times, the medium was removed and the cell monolayer was washed four times with D-PBS. Cells were detached from the plate by incubation with 0.5 ml of 0.25% trypsin solution for 20 min. Cells were placed in a 20-ml scintillation vial; each well of the plate was washed with 0.5 ml of D-PBS and combined with the cells. The amount of radioactivity associated with the cells was determined by counting in a liquid scintillation counter, with 10 ml of scintillation cocktail.

Cellular localization of antisense oligonucleotide. HUVEC were grown on fibronectin-coated glass microscopes slides (5 μg/cm^2). Cells were treated with FITC-labeled oligonucleotide in the presence or absence of DOTMA, as described above. At the indicated times, cells were washed four times with D-PBS and fixed with 2% formaldehyde in D-PBS for 20 min at 25°. After fixation, cells were washed three

times with D-PBS and mounted in glycerol mounting solution (10 mM phosphate, pH 8.5, 150 mM NaCl, 70% glycerol). The subcellular localization of the FITC-labeled oligonucleotide was determined by fluorescent microscopy, using a Nikon Optiphot-2 fluorescent microscope.

Results

Enhanced antisense oligonucleotide activity in the presence of DOTMA. ISIS 1570, a phosphorothioate antisense oligonucleotide that is 18 bases in length (sequence of TGGGAGCCATAGCGAGGC), was designed to hybridize to the human ICAM-1 AUG translation initiation codon and was used to inhibit IL-1β-induced ICAM-1 expression in HUVEC (25). Preincubation of HUVEC for 8 hr with increasing concentrations of ISIS 1570 (from 0.01 to 100 μM) before IL-1β addition to the cells failed to inhibit IL-1β-induced ICAM-1 expression (Fig. 1). Cells pretreated for 4 hr with ISIS 1570 in the presence of 10 μg/ml DOTMA solution (8 μM DOTMA), followed by an additional 4-hr pretreatment period in the absence of DOTMA, before addition of IL-1β (8-hr total pretreatment period) exhibited a concentration-dependent decrease in ICAM-1 expression (Fig. 1). Maximal inhibition occurred between 0.1 and 0.3 μM oligonucleotide (Fig. 1). The inhibition of ICAM-1 expression by ISIS 1570 was demonstrated to be sequence specific, as well as specific for ICAM-1 expression (25). The presence of 8 μM DOTMA solution during the 4-hr pretreatment period enhanced the potency of ISIS 1570 >1000-fold. Addition of Trypan blue to the cells during the first 5 min or last 5 min of treatment with DOTMA did not reveal an increased permeability of the cells to the dye (data not shown). Thus, DOTMA treatment does not promote a generalized increase in cellular permeability to all molecules.

Enhancement by DOTMA of oligonucleotide association with cells. The effect of DOTMA solution on cellular uptake of ISIS 1570 was determined using ISIS 1570 uniformly labeled with ^{35}S (28). The use of uniformly labeled oligonucleotide circumvents many of the problems associated with oligonucleotides labeled at the 5′ terminus, such as removal of the label by phosphatases (27, 32). In the absence of DOTMA, ISIS 1570 associated with the cells in a concentration-dependent manner (Fig. 2), as previously observed with other phosphorothioate oligonucleotides (29). Cells incubated with ^{35}S-ISIS 1570 in the presence of 8 μM DOTMA exhibited between 6- and 18-fold more ^{35}S-ISIS 1570 associated with the cell at each concentration examined. The plateau observed in the presence of DOTMA could be due to saturation of the positively charged lipid species with the negatively charged oligonucleotide. These data demonstrate that the cationic lipid DOTMA enhanced oligonucleotide association with cells at all oligonucleotide concentrations examined.

The optimum concentration of DOTMA was determined by incubating cells for 4 hr in the presence of increasing concentrations of DOTMA plus 0.1 μM ISIS 1570, in the absence of serum. ICAM-1 expression was determined by ELISA, 16 hr after IL-1β addition to the cells. Inhibition of ICAM-1 expression by the antisense oligonucleotide was dependent upon the concentration of DOTMA used (Fig. 3). Concentrations of DOTMA of 24 μM or greater resulted in significant toxicity of the cells, as analyzed by loss of cells from the plate after the overnight incubation. Inhibition of ICAM-1 expression was not due to the presence of DOTMA alone, because there was not a significant difference in ICAM-1 expression between control cells and cells incubated in the presence of up to 16 μM DOTMA (28)(data not shown). Cellular association of ^{35}S-ISIS 1570 was determined in the presence of increasing concentrations of DOTMA (Fig. 3). In agreement with the data examining ICAM-1 expression, increasing concentrations of DOTMA resulted in increased amounts of ^{35}S-ISIS 1570 associated with the cells. Cellular toxicity limited the amount of DOTMA that could be

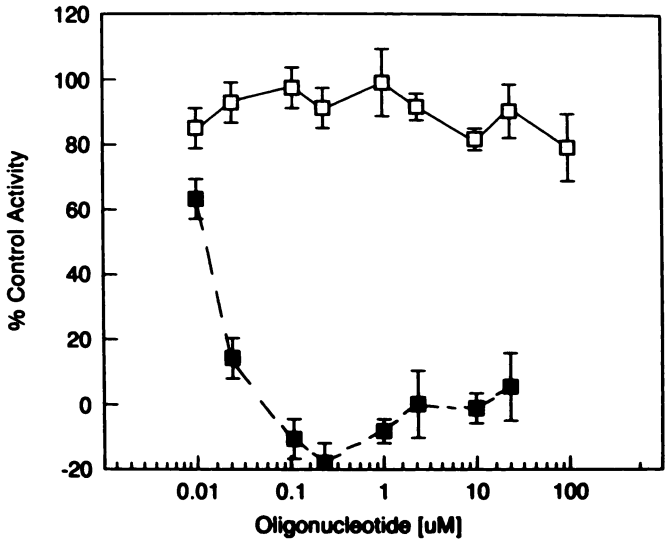

Fig. 1. Effect of DOTMA on antisense oligonucleotide activity. HUVEC were treated for 4 hr with increasing concentrations of the ICAM-1 antisense oligonucleotide ISIS 1570, either in the presence of 8 μM DOTMA (■) or in the absence of DOTMA (□). The medium was replaced with EGM-UV plus oligonucleotide, and cells were incubated an additional 4 hr before stimulation with 5 units/ml IL-1β. ICAM-1 expression was determined 16 hr after addition of IL-1β to the cells, by ELISA, as described in Materials and Methods. Data represent mean ± standard error of one experiment (three determinations). Similar results were obtained in three separate experiments.

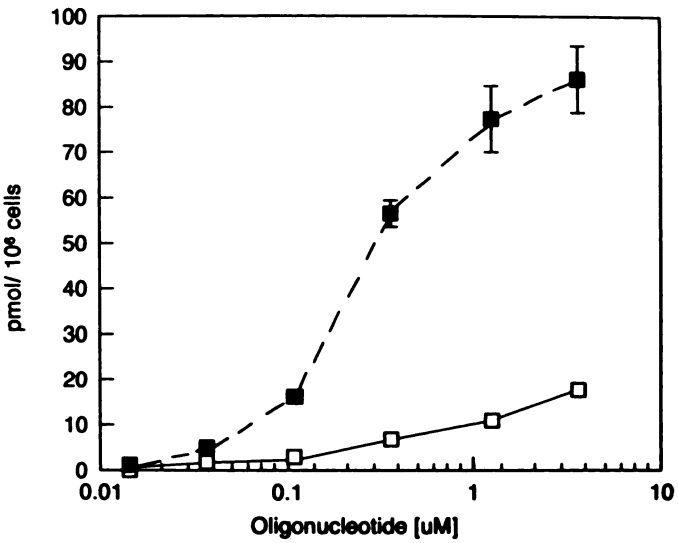

Fig. 2. Effect of DOTMA on cellular association of ^{35}S-ISIS 1570. HUVEC were treated for 4 hr with increasing concentrations of ^{35}S-ISIS 1570, in the absence (□) or presence (■) of 8 μM DOTMA. Cells were washed four times with D-PBS, and the amount of ^{35}S-ISIS 1570 associated with the cells was determined by counting in a liquid scintillation counter. Data represent mean ± standard error (four determinations) of one experiment. Similar results were obtained in a second experiment.

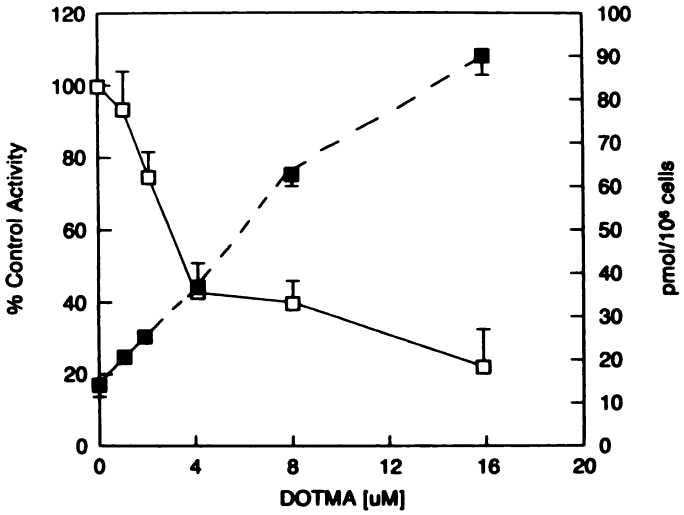

Fig. 3. Effect of increasing concentrations of DOTMA on activity of ISIS 1570. HUVEC were incubated with 100 nM ISIS 1570, in the presence of increasing concentrations of DOTMA, for 4 hr. Medium was replaced with serum-containing medium plus oligonucleotide for an additional 4 hr. Cells were stimulated with 5 units/ml IL-1β for 16 hr, and the level of ICAM-1 expression was determined by ELISA (□). HUVEC were incubated with 1 μM ^{35}S-ISIS 1570 in the presence of increasing concentrations of DOTMA for 4 hr. The amount of ^{35}S-ISIS 1570 associated with the cells was determined as described in Materials and Methods (■). Results represent mean ± standard error (four determinations).

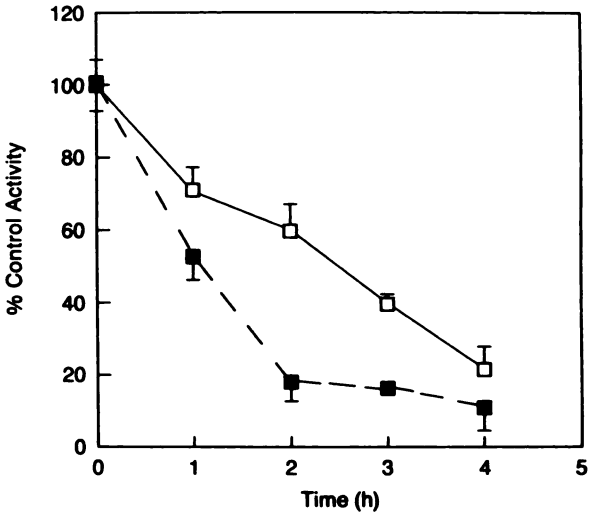

Fig. 4. Effect of treatment time with DOTMA on ISIS 1570 inhibition of ICAM-1 expression. HUVEC were incubated with either 0.1 μM (□) or 1 μM (■) ISIS 1570, in the presence of 8 μM DOTMA, for the indicated period of time. At the indicated time, the cell growth medium was replaced with EGM-UV plus oligonucleotide. The cells were incubated an additional 4 hr and then stimulated with 5 units/ml IL-1β for 16 hr. ICAM-1 expression was determined by ELISA, as described in Materials and Methods. Each data point represents mean ± standard error (three determinations).

added to the cells; therefore, it was not possible to determine whether cellular association of the oligonucleotide reached a plateau with respect to increasing concentrations of DOTMA. For HUVEC, we have used DOTMA at a concentration of 8 μM for the remaining studies; this concentration was a compromise between efficacy and toxicity.

Kinetics of DOTMA-enhanced oligonucleotide effects. The length of treatment time with DOTMA required to observe antisense activity was determined. Cells were incubated for various periods of time with either 0.1 μM or 1 μM ISIS 1570, in the presence of 8 μM DOTMA. Optimal effects were observed after 2-hr treatment with DOTMA at 1 μM ISIS 1570 (Fig. 4). An equivalent level of inhibition was obtained by 4 hr with 0.1 μM ISIS 1570 (Fig. 4). The kinetics of oligonucleotide cellular association over a 6-hr time period were measured in the absence and presence of 8 μM DOTMA. In agreement with the antisense activity, DOTMA markedly enhanced the rate of cellular association of ISIS 1570 (Fig. 5). The right *y*-axis of Fig. 5 corresponds to the amount of oligonucleotide associated with cells in the absence of DOTMA. The kinetics of cellular association appeared to be biphasic, both in the presence and in the absence of DOTMA (Fig. 5). Similar results were reported for ISIS 1082, a phosphorothioate oligonucleotide that targets herpes virus (29). The two phases were probably due to rapid binding of phosphorothioate oligonucleotide to the cell surface followed by internalization of the oligonucleotide. Extrapolating the second phase of cellular association to fit a straight line, the rate of oligonucleotide association with cells in the absence of DOTMA was 0.4 pmol of oligonucleotide/10^6 cells/hr and in the presence of DOTMA was 10.4 pmol of oligonucleotide/10^6 cells/hr, a 25-fold increase in rate (4000 molecules of oligonucleotide/min/cell in the absence of DOTMA and 104,000 molecules of oligonucleotide/min/cell in the presence of DOTMA). These data demonstrate that

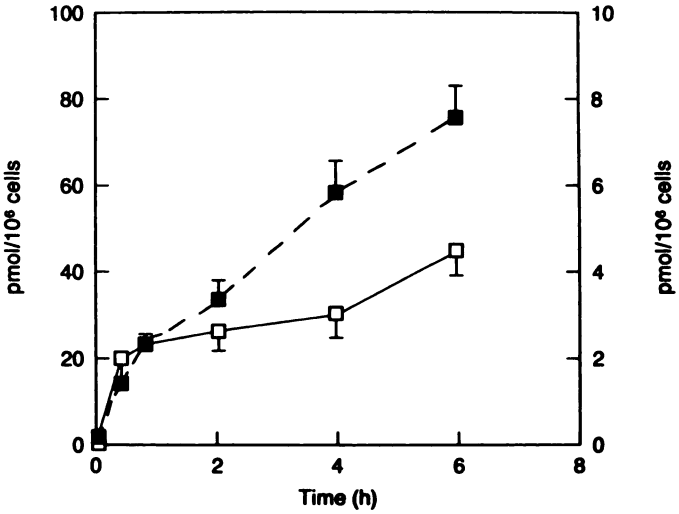

Fig. 5. Kinetics of DOTMA-enhanced oligonucleotide association with cells. HUVEC were incubated with 1 μM ^{35}S-ISIS 1570, in the absence (■) or presence (□) of 8 μM DOTMA. At the indicated times, cells were harvested, and the amount of ^{35}S-ISIS 1570 associated with the cells was determined, as described in Materials and Methods. The scale of the right *y*-axis corresponds to the data obtained in the absence of DOTMA. Each point represents mean ± standard error (four determinations).

DOTMA enhances oligonucleotide cell association in a time- and concentration-dependent manner.

To determine whether the 4-hr incubation time in the presence of DOTMA was sufficient to obtain the antisense effects, cells were incubated for 4 hr in the presence of 8 μM DOTMA and increasing concentrations of ISIS 1570, afterwards the medium was replaced with serum-containing medium in the presence or absence of antisense oligonucleotide. After 16-hr treatment with 5 units/ml IL-1β, the level of ICAM-1 expression was determined by ELISA. ICAM-1 expression was inhib-

ited only 5–10% more if the oligonucleotide was readded to the cells after the initial 4-hr treatment in the presence of DOTMA (Fig. 6). These data suggest that the 4-hr treatment period with DOTMA was sufficient to inhibit expression of ICAM-1 and further incubation with the oligonucleotide after DOTMA treatment did not significantly enhance the activity of the oligonucleotide.

Subcellular distribution of ISIS 1570 in the presence and absence of DOTMA. It should be noted that, whereas DOTMA enhanced the biological activity of the antisense oligonucleotides by at least 1000-fold, cellular association increased only 17-fold. One possible explanation for this discrepancy was that DOTMA changed the subcellular distribution of the oligonucleotide. To evaluate the intracellular distribution of ISIS 1570, the oligonucleotide was labeled with FITC and the distribution of the FITC-labeled oligonucleotide was analyzed by fluorescence microscopy. Incubation of cells with 1 μM FITC-labeled ISIS 1570 for 4 hr resulted in a faint punctate distribution of the oligonucleotide within the cytoplasm of cells (Fig. 7, B and C). Examination of the cells at higher magnification demonstrated that the FITC-labeled oligonucleotide was associated with cytoplasmic structures similar in size and distribution to lysosomes (Fig. 7, D and E). The localization of FITC-labeled ISIS 1570 in endosome/lysosome-like structures is in agreement with other reports, which suggested that a major route of cellular accumulation of oligonucleotides was by endocytosis (15, 30).

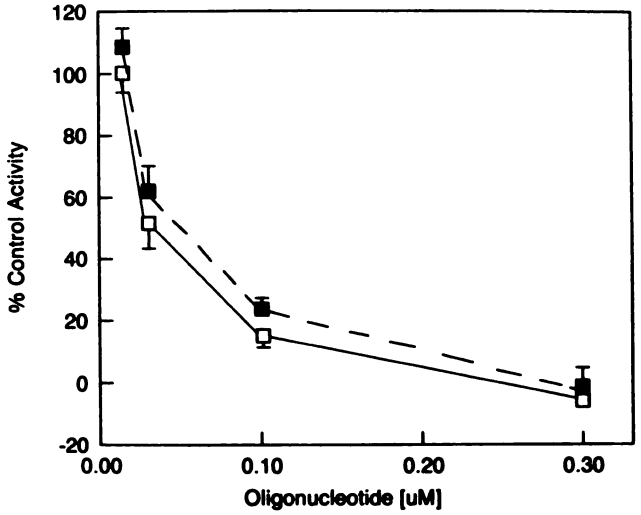

Fig. 6. Effect of readdition of ISIS 1570 to cell culture medium after DOTMA treatment. HUVEC were incubated with increasing concentrations of ISIS 1570, in the presence of 8 μM DOTMA, for 4 hr. Medium was replaced with EGM-UV (■) or EGM-UV plus indicated concentrations of ISIS 1570 (□) for 4 hr before stimulation with 5 units/ml IL-1β for 16 hr. ICAM-1 expression was determined by ELISA, as described in Materials and Methods. Results are mean ± standard error (three determinations).

Cells incubated with 1 μM FITC-labeled ISIS 1570 in the presence of DOTMA exhibited bright nuclear fluorescence with some cytoplasmic staining, primarily as bright punctate structures (Fig. 7, F, G, H, and I). The FITC-labeled oligonucleotide was excluded from nucleoli and appeared to be concentrated within specific domains of the nucleus (Fig. 7, H and I). The data obtained with the use of FITC-labeled oligonucleotide agreed with those obtained using ^{35}S-labeled oligonucleotide, in that DOTMA significantly enhanced the amount of oligonucleotide associated with cells. To emphasize this point, the cells shown in Fig. 7B, incubated in the absence of DOTMA, were photographed at the same exposure as those in Fig. 7G, incubated in the presence of DOTMA (8 sec). The exposure time was increased from 8 sec to 72 sec for visualization of the localization of the oligonucleotide in Fig. 7C. These data demonstrate that DOTMA not only enhanced cellular association of oligonucleotides but also markedly changed the intracellular distribution of the oligonucleotide. In the absence of DOTMA the oligonucleotide appeared to have localized in endosome vesicles and/or lysosomes, whereas in the presence of DOTMA the oligonucleotide was predominantly localized in the nucleus.

The localization of the oligonucleotide in specific cellular compartments was not due to the fluorescein dye, because oligonucleotides labeled with rhodamine or biotin, followed by FITC-labeled streptavidin, gave identical results (data not shown). Furthermore, the observed fluorescence was not due to free fluorescein released after degradation of the oligonucleotide, because cells incubated with 1 μM fluorescein exhibited very weak fluorescence that was not localized to discrete structures. In addition, our data, as well as others' (31, 32), suggest that phosphorothioate oligonucleotides are stable under the conditions used for these experiments. Similar intracellular localization of FITC-labeled ISIS 1570 was observed in unfixed cells, so the intracellular distribution of the oligonucleotide was not an artifact of the fixation conditions.

The intracellular localization of FITC-labeled oligonucleotide was examined at various times after addition to cells. As early as 15 min after addition of oligonucleotides in the presence

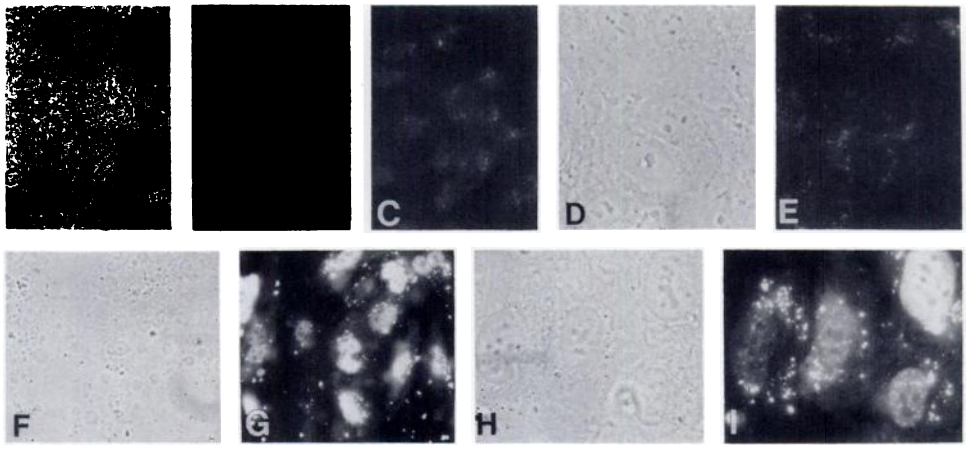

Fig. 7. Subcellular localization of FITC-labeled ISIS 1570. HUVEC were grown on fibronectin-treated glass slides. Cells were incubated with 1 μM FITC-labeled ISIS 1570 (A, B, C, D, and E) or 1 μM FITC-labeled ISIS 1570 plus 8 μM DOTMA (F, G, H, and I) for 4 hr at 37°. Cells were washed four times with D-PBS, fixed with 2% formaldehyde, and observed by phase contrast (A, D, F, and H) or fluorescence microscopy (B, C, E, G, and I), as described in Materials and Methods.

of DOTMA, 5–10% of the cells exhibited weak nuclear fluorescence (data not shown). Examination of live cells in the presence of FITC-labeled ISIS 1570 and DOTMA revealed that the FITC-labeled oligonucleotide first distributed diffusely throughout the cytoplasm and then accumulated in the nucleus (data not shown). These data suggest that, in the presence of DOTMA, the oligonucleotide enters the cell through the cytoplasm, rather than being transported directly into the nucleus. By 1 hr after addition of oligonucleotides to cells in the presence of DOTMA, the percentage of cells exhibiting nuclear fluorescence increased to 65% (Fig. 8, A and B). At 1 hr most cells also exhibited diffuse cytoplasmic fluorescence, as well as strong punctate cytoplasmic fluorescence (Fig. 8B). The percentage of cells exhibiting nuclear fluorescence increased to 70–80% 4 hr after addition of oligonucleotide in the presence of DOTMA (Fig. 8, E and F). The fluorescence intensity per cell also increased with time. The FITC-labeled oligonucleotide appeared to localize predominantly in nuclei and as bright punctate structures in the cytoplasm (Fig. 8, E and F). Eight hours after addition of oligonucleotide to cells (4 hr in the presence of DOTMA and 4 hr in the absence of DOTMA), the oligonucleotide remained associated with nuclei and in punctate structures within the cytoplasm (Fig. 8, I and J). After 24 hr (4 hr in the presence of DOTMA and 20 hr in the absence of DOTMA), fewer cells exhibited nuclear fluorescence and the fluorescence intensity within nuclei was decreased (Fig. 8, M and N). However, the cytoplasmic structures that accumulated the oligonucleotide did not show a marked loss of fluorescence intensity.

In the absence of DOTMA, there was not a marked change in the subcellular distribution at any of the times examined. At early times (15 min), the oligonucleotide appeared to be predominantly localized on the cell surface (data not shown). At 1, 4, 8, and 24 hr, the oligonucleotide exhibited a punctate distribution within the cytoplasm (Fig. 8, C and D, G and H, K and L, O and P, respectively). At no time investigated (up to 48 hr) did we find evidence of nuclear accumulation of oligonucleotide in the absence of DOTMA. The fluorescence intensity of the punctate cytoplasmic structures increased with increasing times of oligonucleotide incubation. The cytoplasmic structures with which the oligonucleotide associated in the absence of DOTMA were smaller than the cytoplasmic struc-

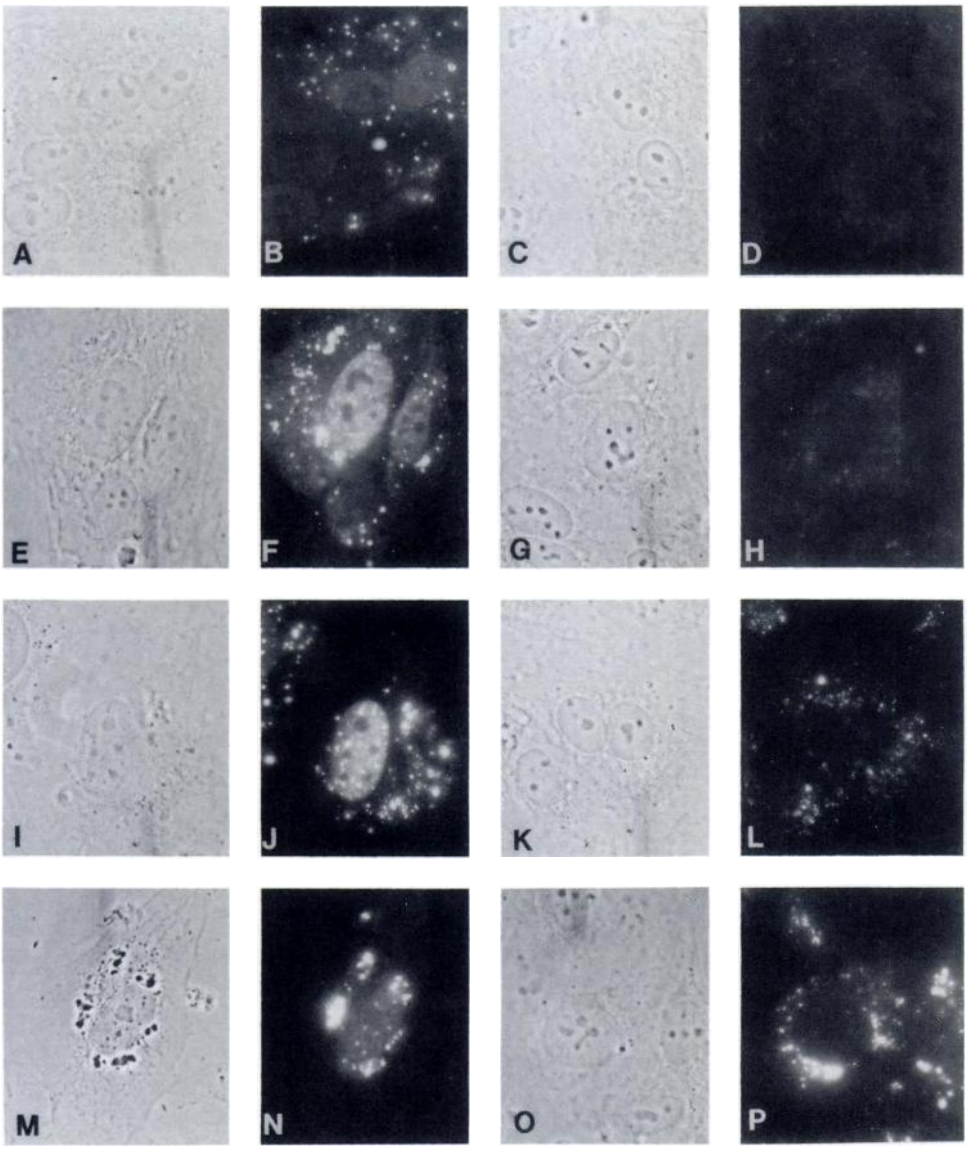

Fig. 8. Kinetics of oligonucleotide localization in the cell nucleus. HUVEC grown on glass microscope slides were treated with 1 μM FITC-labeled ISIS 1570, in either the presence (A, B, E, F, I, J, M, and N) or absence (C, D, G, H, K, L, O, and P) of 8 μM DOTMA. At 1 hr (A, B, C, and D), 4 hr (E, F, G, and H), 8 hr (I, J, K, and L), or 24 hr (M, N, O, and P) after the addition of the oligonucleotide to the cells, the cells were washed and fixed as described in Materials and Methods.

tures accumulating FITC-labeled oligonucleotide in the presence of DOTMA (<1 μm versus 1–4 μm). It is not clear whether these represent distinct cytoplasmic structures or whether the DOTMA mixture promotes fusion of the smaller cytoplasmic vesicles.

To better understand the mechanism by which DOTMA enhances cellular uptake of oligonucleotide and nuclear accumulation, the effects of various inhibitors of endocytosis on the subcellular localization of the oligonucleotide were examined in the presence and absence of DOTMA. Incubation of the cells at 4° in the absence or presence of DOTMA completely inhibited accumulation of the FITC-labeled oligonucleotide within cells (data not shown). Cells incubated at 4° did exhibit fluorescence on the cell surface, suggesting that the oligonucleotide was still capable of binding but was not internalized. The lysosomotropic agent chloroquine did not significantly change nuclear accumulation of FITC-labeled ISIS 1570 (Fig. 9, C and D). In agreement with this result, incubation of HUVEC with 100 μM chloroquine did not modify the sensitivity of the cells to inhibition of ICAM-1 expression with ISIS 1570 (data not shown). Examination of the cells under phase-contrast microscopy revealed that cells treated with 100 μM chloroquine exhibited large vacuoles in the cytoplasm of the cell (Fig. 9E). In the absence of DOTMA the oligonucleotide accumulated in cytoplasmic structures that were larger in size than those in untreated cells (Fig. 9F). Cells shown in Fig. 9, E and F, were incubated with 5 μM FITC-labeled ISIS 1570, to enhance visualization of the cytoplasmic structures in which the oligonucleotide accumulated. Similar results were obtained with 20 mM NH_4Cl (data not shown).

Microtubules are involved in the later stages of endocytosis (33, 34). Cells were pretreated for 30 min with the microtubule-depolymerizing agent nocodazole (33 μM) before the addition of oligonucleotides. Nocodazole reduced the amount of oligonucleotide accumulated in the nucleus, as determined by fluorescence intensity (Fig. 9G); however, the percentage of cells exhibiting nuclear fluorescence did not change. The number of cytoplasmic structures containing fluorescent oligonucleotide was also decreased in nocodazole-treated cells, and the structures were localized in the cell periphery rather than the perinuclear area. In the absence of DOTMA, nocodazole treatment of cells did not markedly change the distribution of the oligonucleotide (data not shown). Similar results were also obtained with 1 μM colcemid (data not shown). Immunolocalization of tubulin demonstrated that these concentrations of nocodazole and colcemid promoted depolymerization of microtubules in HUVEC (data not shown). Brefeldin A is a fungal metabolite that interferes with membrane recycling between the endoplasmic reticulum and *cis* Golgi (35). Pretreatment of HUVEC with 1 μg/ml brefeldin A did not markedly change the distribution of oligonucleotide in the presence (Fig. 9, I and J) or absence (data not shown) of DOTMA.

The monovalent carboxylic ionophore monensin has been demonstrated to block receptor-mediated endocytosis of molecules and virus particles (36, 37). Treatment of cells with 5 μM monensin almost completely eliminated nuclear accumulation of FITC-labeled ISIS 1570 in the presence of DOTMA (Fig. 9, K and L). Oligonucleotide still accumulated in the cytoplasmic vesicles in the presence of monensin (Fig. 9, K and L). In the absence of DOTMA, monensin did not markedly change the cellular distribution of the oligonucleotide (data not shown).

Discussion

Numerous studies have demonstrated that antisense oligonucleotides, containing either phosphodiester internucleosidic linkages, phosphorothioate linkages, or methylphosphonate-linkages, inhibit expression of viral and host gene products (1–13). It is inferred from these studies that oligonucleotides are capable of entering cells and achieve sufficient concentrations, within the same cellular compartment as the target mRNA or pre-mRNA, to inhibit expression of target protein. Several laboratories have investigated the mechanism of cellular uptake of oligonucleotides (14, 15, 29, 30). These studies suggest that phosphodiester and phosphorothioate oligonucleotides enter cells by an endocytotic process, whereas methylphosphonates enter cells by passive diffusion (14, 15). These conclusions were based upon concentration, temperature, and energy dependence of oligonucleotide uptake, binding of phosphodiester oligonucleotides to a specific membrane protein, and localization of fluorescent probe-labeled oligonucleotides in a punctate pattern within the cytoplasm of the cells, presumably in lysosomes. Although these studies by no means unequivocally demonstrated that phosphodiester- and phosphorothioate-containing oligonucleotides enter cells by endocytosis, the data are consistent with these conclusions. It was not clear from these studies how phosphodiester or phosphorothioate oligonucleotides leave internalized membrane structures to become free in the cytoplasm or nucleus, to interact with target mRNA.

We were unable to detect activity with antisense oligonucleotides directed against the AUG translation initiation codon of ICAM-1, using phosphodiester or phosphorothioate oligonucleotides added directly to the growth medium of cells, in either the presence or absence of serum. The lack of activity of the ICAM-1 antisense oligonucleotide in the absence of DOTMA was not unique to HUVEC. We also failed to obtain antisense activity with the ICAM-1 oligonucleotide in A549 cells, A431 cells, primary human keratinocytes, and HeLa cells in the absence of DOTMA. However, in the presence of DOTMA we observed inhibition of ICAM-1 expression in each of the cell lines. The optimum ratio of DOTMA to oligonucleotide varied as a function of the cell line. Preliminary data suggest that DOTMA may not enhance oligonucleotide uptake into all types of cells, because cellular uptake of FITC-labeled ISIS 1570 was unaffected by the presence of DOTMA in HL-60 and RBL-1 cells.

Most studies that report inhibition of gene expression with antisense oligonucleotides target either viral proteins or cellular proteins thought to be involved in cellular proliferation. In the case of the viral targets, some of the inhibition of viral gene products could be due to nonspecific effects of the oligonucleotides on reverse transcriptase, DNA polymerase, or viral binding to the cell membrane (1–4, 38). Additionally, the enveloped viruses, such as herpes, may markedly change the intracellular distribution of the oligonucleotide in the infected cell, such that the oligonucleotide becomes accessible to the target mRNA. Many of the cellular proteins that were inhibited by antisense oligonucleotides were tested in leukocytes or leukocyte-derived cell lines (1–9, 13). It is not known whether leukocyte-derived cells take up oligonucleotide by different mechanisms than do the cells used for our studies. Alternatively, it is possible that proliferation-associated proteins are more sensitive to inhibition with antisense oligonucleotides than are non-proliferation-

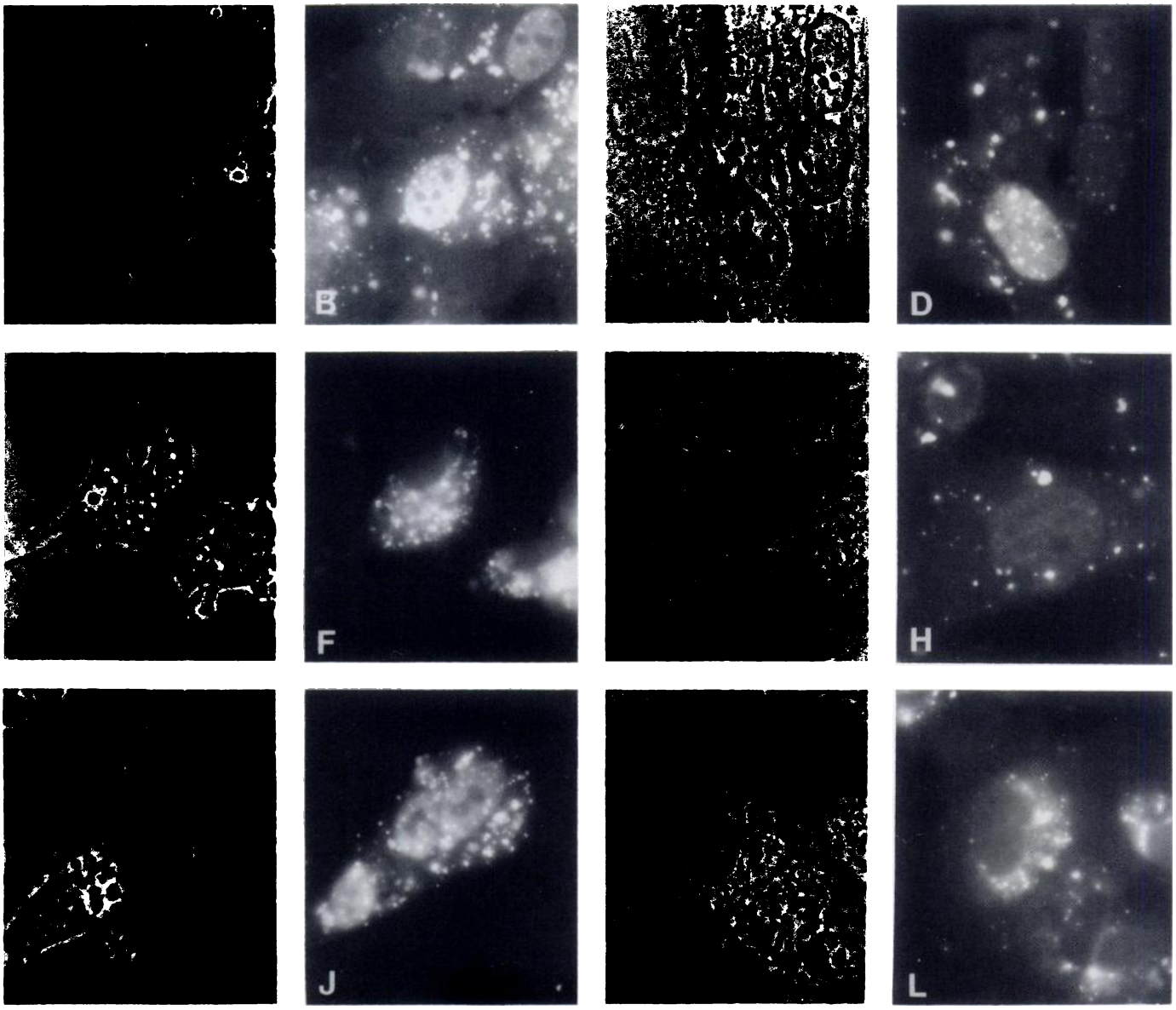

Fig. 9. Effect of inhibitors on localization of FITC-labeled ISIS 1570. HUVEC were grown on glass microscope slides. The inhibitors were added to the cells 20 min before the addition of oligonucleotide and DOTMA. A and B, Phase contrast and fluorescence microscopy of cells incubated for 4 hr in the presence of 1 μM FITC-labeled ISIS 1570 plus 8 μM DOTMA; C and D, phase contrast and fluorescence microscopy of cells incubated with 100 μM chloroquine, 1 μM FITC-labeled ISIS 1570, and 8 μM DOTMA for 4 hr; E and F, phase contrast and fluorescence microscopy of cells incubated with 100 μM chloroquine plus 5 μM FITC-labeled ISIS 1570; G and H, phase contrast and fluorescence microscopy of cells incubated with 33 μM nocodazole, 1 μM FITC-labeled ISIS 1570, and 8 μM DOTMA; I and J, phase contrast and fluorescence microscopy of cells incubated with 1 μg/ml brefeldin A, 1 μM FITC-labeled ISIS 1570, and 8 μM DOTMA; K and L, phase contrast and fluorescence microscopy of cells incubated with 5 μM monensin, 1 μM ISIS 1570, and 8 μM DOTMA.

associated gene products, or there may be different intracellular localization of the target RNAs.

Additionally, it is not clear how the pharmacokinetics of phosphorothioate oligonucleotides in cell culture, in the presence or absence of cationic lipids, relate to those found in tissue, organs, and whole animals. Recent studies in mice (39) have demonstrated that phosphorothioate oligonucleotides, in the absence of any formulation, are distributed broadly in tissues after intravenous and intraperitoneal administration. Our laboratory has also conducted whole-animal pharmacokinetic experiments in mice and rabbits, which suggest that phosphorothioate oligonucleotides are cleared from the blood in a biphasic manner, distribute into a variety of tissues, and are excreted exclusively in the urine.[2] Determination of whether formulation of phosphorothioate oligonucleotides with cationic lipids or other types of lipids alters their *in vivo* pharmacokinetics and pharmacology awaits further studies.

In the presence of the cationic lipid DOTMA, antisense activity with phosphorothioate oligonucleotides was easily detected; natural phosphodiester oligonucleotides failed to exhibit any activity under similar experimental conditions. It is not clear whether the lack of activity with phosphodiester oligonucleotides was due to their increased nuclease sensitivity or to a lack of interaction with DOTMA-containing vesicles. With DOTMA-containing vesicles, it was possible to demonstrate

[2] Unpublished observations.

specific antisense activity with phosphorothioate oligonucleotides targeting several regions of the ICAM-1 mRNA (25). DOTMA-containing lipid vesicles increased both the rate of oligonucleotide association with cells and, as a result, the extent of oligonucleotide association with cells.

DOTMA-containing lipid vesicles not only enhanced the rate of oligonucleotide uptake into cells but also markedly changed the subcellular distribution of the oligonucleotide. In the absence of DOTMA, the FITC-labeled oligonucleotide appeared to be associated with cytoplasmic structures consistent with endosomal or lysosomal vesicles, based upon a punctate perinuclear cytoplasmic fluorescence. These findings are in agreement with other reports examining the cellular distribution of oligonucleotides (15, 30, 40). The major difference in the distribution of the oligonucleotide in the presence of DOTMA was the localization of the oligonucleotide in the cell nucleus. The cytoplasmic distribution of the oligonucleotides in the presence of DOTMA also appeared to be somewhat different, in that the structures that accumulated oligonucleotide were larger in size. At the light microscopic level, it was not possible to identify definitively whether the oligonucleotide was contained within the cytoplasmic vesicles or associated with the surface of the vesicles. Kinetic experiments suggest that, in the presence of DOTMA, the oligonucleotide first enters the cell through the cytoplasm and then accumulates in the nucleus. It is unlikely that accumulation of the phosphorothioate oligonucleotide in the nucleus was due to trafficking into the nucleus promoted by DOTMA. However, it is not known whether DOTMA remains associated with the oligonucleotide within the nucleus. Previous studies have demonstrated nuclear localization of phosphorothioate and phosphodiester oligonucleotides microinjected into the cytoplasm of the cell (41, 42). Accumulation within the nucleus appears to be mediated by diffusion through nuclear pores, because it was not affected by ATP depletion (41, 42). Thus, liposomes composed of the cationic lipid DOTMA appear to be an efficient method for delivery of oligonucleotides into the cytoplasm of the cell, similar to direct microinjection. The advantage of DOTMA-containing vesicles is that oligonucleotides can be introduced into large numbers of cells.

The fact that phosphorothioate and phosphodiester oligonucleotides appear to concentrate within the nucleus after introduction into the cytoplasm may have important ramifications for mechanisms of action of antisense oligonucleotides. We found that modified oligonucleotides that support RNase H activity were more effective in inhibiting ICAM-1 expression (25). RNase H has been reported to be a nuclear enzyme (43); thus, the oligonucleotides are localized in the same cellular compartment. Chin *et al.* (41) also reported that phosphodiester and, to a lesser extent, phosphorothioate oligonucleotides colocalize to Sm-staining structures. Sm is an autoantibody that recognizes a common epitope on U1, U2, U4, U5, and U6 small nuclear ribonucleoprotein particles, which are thought to be involved in pre-mRNA splicing (44). Thus, the oligonucleotides may concentrate to areas of the nucleus where RNA processing occurs. It is possible that high concentrations of oligonucleotides in regions of the cell nucleus where RNA processing is thought to occur may have undesired effects. However, at the oligonucleotide concentrations used for our studies, we have been able to demonstrate specific inhibition of the target protein (25).[1]

The mechanism by which DOTMA-containing lipid vesicles enhance oligonucleotide uptake has not been entirely elucidated. DOTMA vesicles are a 1:1 mixture of DOTMA and dioleoyl phosphatidylethanolamine, which form 250-nm liposomes (45). DNA is thought to bind initially to the surface of the liposome by ionic interactions. The DOTMA-containing vesicles interact with the cell membrane, possibly by initial ionic interactions. Agents that increase lysosomal pH, such as chloroquine and NH_4Cl, do not prevent accumulation of the antisense oligonucleotide within the cell nucleus or block antisense activity (data not shown). These results suggest that acidification of the endosomal vesicles was not required for release of the oligonucleotide into the cytoplasm; thus, these lipid vesicles do not behave as pH-sensitive liposomes. Furthermore, microtubule-depolymerizing agents do not prevent nuclear accumulation of the oligonucleotide; thus, nuclear accumulation was not dependent upon microtubules. Monensin, a Na^+ ionophore that disrupts Na^+ and proton gradients across biological membranes (36, 37), distinguishes nuclear versus cytoplasmic localization of oligonucleotides in DOTMA-treated cells. In the presence of monensin, the accumulation of oligonucleotide in the cell nucleus was markedly reduced, whereas accumulation in cytoplasmic vesicles was unchanged. The effects of monensin were probably not due to increasing the pH of endocytotic vesicles, because chloroquine and NH_4CL had no effect on antisense activity or nuclear accumulation of oligonucleotides. Thus, monensin may prevent fusion of DOTMA vesicles with the plasma membrane. From these experiments, it is not known whether the effect of monensin is directly on the DOTMA vesicle or on the cell. The calcium ionophore A23187 did not prevent accumulation of oligonucleotide within the cell nucleus (data not shown); therefore, it is unlikely that the effects of monensin were due solely to increasing the permeability of DOTMA vesicles to ions.

In addition to enhancing and modifying distribution of the oligonucleotides, cationic lipids may also enhance antisense activity by increasing the rate at which the oligonucleotide hybridizes to its target mRNA. Pontius and Berg (46) recently reported that cationic detergents, such as dodecyltrimethylammonium bromide and cetyltrimethylammonium bromide, enhance the rate of renaturation of complementary DNA strands by as much as 10^4-fold. In addition, the cationic detergents stabilized the duplex to thermal denaturation. DOTMA, as well as the detergents used by Pontius and Berg (46), contain a quaternary amine and long aliphatic groups. Thus, it would be predicted that DOTMA and other cationic lipids would exhibit similar activity. Experiments are currently in progress to address what role enhanced hybridization kinetics play in the activity of cationic lipids.

Covalent attachment of lipophilic molecules to oligonucleotides, such as cholesterol, undecyl, dodecanol, and triethylammonium, 1,2-di-*O*-hexadecyl-*rac*-glycerol-^{3}H-phosphate, has been performed to enhance cellular uptake of oligonucleotides (40, 47–49). In addition, conjugation of poly-L-lysine or poly-L-lysine plus heparin to oligonucleotides, to neutralize the negative charge of the phosphate backbone, has been reported (50, 51). In many instances, the increase in activity was not marked or the oligonucleotides lacked sequence specificity (48–50, 52). Furthermore, conjugation methods are time consuming and not amenable to screening of large numbers of molecules. Liposomes have been used to enhance c-*myc*-containing anti-

sense oligonucleotides (53). In the case of liposomes, the oligonucleotide had to be encapsulated before addition to cells and may be restricted to only a few cell types, such as phagocytic cells. These results demonstrate that cationic lipids such as DOTMA enhance accumulation of oligonucleotides in a number of cell types, resulting in a marked enhancement of antisense activity. Using DOTMA-containing vesicles, we have been able to demonstrate specific antisense activity against a number of targets, including ICAM-1, and have been able to identify rapidly the optimum target site on a mRNA for antisense oligonucleotides (25). DOTMA has an advantage over other cell culture delivery methods, in that no preformulation of the liposome with the oligonucleotide was required. The main disadvantages of DOTMA are toxicity and markedly decreased activity in the presence of serum. Newer cationic lipid formulations are available that reportedly exhibit decreased toxicity (54, 55). Thus, cationic lipids have proven to be useful for delivery of antisense oligonucleotides into cells in culture and potentially could be of use for the delivery of antisense oligonucleotides into animals.

Acknowledgments

The authors would like to thank Ms. Mary Ann Zounes for oligonucleotide synthesis and Drs. Stanley T. Crooke, David Ecker, P. Dan Cook, and Rosanne Crooke for many helpful discussions.

References

1. Stein, C. A., and J. S. Cohen. Oligodeoxynucleotides as inhibitors of gene expression: a review. *Cancer Res.* **48**:2659–2668 (1988).
2. Zon, G. Oligonucleotide analogues as potential chemotherapeutic agents. *Pharm. Res.* **5**:539–549 (1989).
3. Izant, J. G. Antisense "pseudogenetics." *Cell Motility Cytoskeleton* **14**:81–91 (1989).
4. Helene, C., and J.-J. Toulme. Specific regulation of gene expression by antisense, sense and antigene nucleic acids. *Biochim. Biophys. Acta* **1049**:99–125 (1990).
5. Heikkila, R., G. Schwab, E. Wickstrom, S. L. Loke, D. H. Pluznik, R. Watt, and L. M. Neckers. A c-*myc* antisense oligodeoxynucleotide inhibits entry into S phase but not progress from G_0 to G_1. *Nature* (*Lond.*) **328**:445–449 (1987).
6. Gewirtz, A. M., and B. Calabretta. A c-*myb* antisense oligonucleotide inhibits normal human hematopoiesis *in vitro*. *Science* (*Washington D. C.*) **242**:1303–1306 (1988).
7. Holt, J. T., R. L. Redner, and A. W. Nienhuis. An oligomer complementary to c-*myc* mRNA inhibits proliferation of HL-60 promyelocytic cells and induces differentiation. *Mol. Cell. Biol.* **8**:963–973 (1988).
8. Harel-Bellan, A., S. Durum, K. Muegge, A. K. Abbas, and W. L. Farrar. Specific inhibition of lymphokine biosynthesis and autocrine growth using antisense oligonucleotide in Th1 and Th2 helper T cell clones. *J. Exp. Med.* **168**:2309–2318 (1988).
9. Birchenall-Roberts, M. C., C. Ferrer, D. Ferris, L. A. Falk, J. Kasper, G. White, and F. W. Ruscetti. Inhibition of murine monocyte proliferation by a colony-stimulating factor-1 antisense oligodeoxynucleotide: evidence for autocrine regulation. *J. Immunol.* **145**:3290–3296 (1990).
10. Goodarzi, G., S. C. Gross, A. Tewari, and K. Watabe. Antisense oligodeoxyribonucleotides inhibit expression of the gene for hepatitis B surface antigen. *J. Gen. Virol.* **71**:3021–3025 (1990).
11. Florini, J. R., and D. Z. Ewton. Highly specific inhibition of IGF-I-stimulated differentiation by an antisense oligodeoxyribonucleotide to myogenin mRNA. *J. Biol. Chem.* **265**:13435–13437 (1990).
12. Maier, J. A. M., P. Voulalas, D. Roeder, and T. Macaig. Extension of the lifespan of human endothelial cells by an interleukin-1α antisense oligomer. *Science* (*Washington D. C.*) **249**:1570–1574 (1990).
13. Szczylik, C., T. Skorski, N. C. Nicolaides, L. Manzella, L. Malaguarnera, D. Venturelli, A. M. Gewirtz, and B. Calabretta. Selective inhibition of leukemia cell proliferation by BCL-ABL antisense oligodeoxynucleotides. *Science* (*Washington D. C.*) **253**:562–565 (1991).
14. Miller, P. S. Oligonucleoside methylphosphonates as antisense reagents. *Biotechnology* **9**:358–362 (1991).
15. Loke, S. L., C. A. Stein, X. H. Zhang, K. Mori, M. Nakanishi, C. Subasinghe, J. S. Cohen, and L. M. Neckers. Characterization of oligonucleotide transport into living cells. *Proc. Natl. Acad. Sci. USA* **86**:3474–3478 (1989).
16. Agrawal, S., S. H. Mayrand, P. C. Zamecnik, and T. Pederson. Site-specific excision from RNA by RNase H and mixed-phosphate-backbone oligodeoxynucleotides. *Proc. Natl. Acad. Sci. USA* **87**:1401–1405 (1990).
17. Osborn, L. Leukocyte adhesion to endothelium in inflammation. *Cell* **56**:907–910 (1990).
18. Springer, T. A. Adhesion receptors of the immune system. *Nature* (*Lond.*) **346**:425–43 (1990).
19. Dustin, M. L., and T. A. Springer. Lymphocyte function-associated antigen-1 (LFA-1) interaction with intercellular adhesion molecule-1 (ICAM-1) is one of at least three mechanisms for lymphocyte adhesion to cultured endothelial cells. *J. Cell Biol.* **107**:321–331 (1988).
20. Rothlein, R., M. Czajkowski, M. M. O'Neil, S. D. Marlin, E. Mainolfi, and V. J. Merluzzi. Induction of intercellular adhesion molecule 1 on primary and continuous cell lines by pro-inflammatory cytokines: regulation by pharmacological agents and neutralizing antibodies. *J. Immunol.* **141**:1665–1669 (1988).
21. Griffiths, C.E., J. J. Voorhees, and B. J. Nickoloff. Characterization of intercellular adhesion molecule-1 and HLA-DR expression in normal and inflamed skin: modulation by recombinant *gamma* interferon and tumor necrosis factor. *J. Am. Acad. Dermatol.* **20**:617–629 (1989).
22. Hale, L. P., M. E. Martin, D. E. McCollum, J. A. Nunley, T. A. Springer, K. H. Singer, and B. F. Haynes. Immunohistologic analysis of the distribution of cell adhesion molecules within the inflammatory synovial microenvironment. *Arthritis Rheum.* **32**:22–30 (1989).
23. Lisby, S., E. Ralfkiaer, R. Rothlein, and G. L. Vejlsgaard. Intercellular adhesion molecule-1 (ICAM-1) expression correlated to inflammation. *Br. J. Dermatol.* **120**:479–484 (1989).
24. Volpes, R., J. J. van den Oord, and V. J. Desmet. Immunohistochemical study of adhesion molecules in liver inflammation. *Hepatology* **12**:59–65 (1990).
25. Chiang, M.-Y., H. Chan, M. A. Zounes, S. M. Freier, W. F. Lima, and C. F. Bennett. Antisense oligonucleotides inhibit intercellular adhesion molecule 1 expression by two distinct mechanisms. *J. Biol. Chem.* **266**:18162–18171 (1991).
26. Felgner, P. L., T. R. Gadek, M. Holm, R. Roman, H. W. Chan, M. Wenz, J. P. Northrup, G. M. Ringold, and M. Danielson. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. USA* **84**:7413–7417 (1987).
27. Iyer, R. P., W. Egan, J. B. Regan, and S. L. Beuacage. ^{3}H-1,2-Benzodithiole-3-one-1,1-dioxide as an improved sulfurizing reagent in the solid phase synthesis of oligodeoxyribonucleoside phosphorothioates. *J. Am. Chem. Soc.* **112**:1253–1254 (1990).
28. Stein, C. A., P. L. Iversen, C. Subasinghe, J. S. Cohen, W. J. Stec, and G. Zon. Preparation of ^{35}S-labeled polyphosphorothioate oligodeoxyribonucleotides by use of hydrogen phosphonate chemistry. *Anal. Biochem.* **188**:11–16 (1990).
29. Crooke, R. M. *In vitro* toxicology and pharmacokinetics of antisense oligonucleotides. *Anti-cancer Drug Design* **6**:609–646 (1992).
30. Yakubov, L. A., E. A. Deeva, V. F. Zarytova, E. M. Ivanova, A. S. Ryte, L. V. Yurchenko, and V. V. Vlassov. Mechanism of oligonucleotide uptake by cells: involvement of specific receptors. *Proc. Natl. Acad. Sci. USA* **86**:6454–6458 (1989).
31. Campbell, J. M., T. A. Bacon, and E. Wickstrom. Oligodeoxynucleoside phosphorothioate stability in subcellular extracts, culture media, sera and cerebrospinal fluid. *J. Biochem. Biophys. Methods* **20**:259–267 (1990).
32. Shaw, J.-P., K. Kent, J. Fishback, and B. Froehler. Modified deoxyoligonucleotides stable to exonuclease degradation in serum. *Nucleic Acids Res.* **19**:747–750 (1991).
33. Bomsel, M., R. Parton, S. A. Kuznetsov, T. A. Schroer, and J. Greunberg. Microtubule- and motor-dependent fusion *in vitro* between apical and basolateral endocytotic vesicles from MDCK cells. *Cell* **62**:719–732 (1990).
34. Kelly, R. B. Microtubules, membrane traffic, and cell organization. *Cell* **61**:5–7 (1990).
35. Misumi, Y., Y. Misumi, K. Miki, A. Takatsuki, G. Tamura, and Y. Ikehara. Novel blockade by brefeldin A of intracellular transport of secretory proteins in cultured rat hepatocytes. *J. Biol. Chem.* **261**:11398–11403 (1986).
36. Pressman, B. C. Biological applications of ionophores. *Annu. Rev. Biochem.* **45**:501–530 (1976).
37. Ledger, P. W., and M. L. Tanzer. Monensin: a perturbant of cellular physiology. *Trends Pharmacol. Sci.* **9**:313–314 (1984).
38. Gao, W.-Y., J. W. Jaroszewski, J. S. Cohen, and Y.-C. Cheng. Mechanisms of herpes simplex virus type 2 growth by 28-mer phosphorothioate oligodeoxycytidine. *J. Biol. Chem.* **265**:21072–20178 (1990).
39. Agrawal, S., J. Temsamani, and J. Y. Tang. Pharmacokinetics, biodistribution, and stability of oligodeoxynucleotide phosphorothioates in mice. *Proc. Natl. Acad. Sci. USA* **88**:7595–7599 (1991).
40. Saison-Behmoaras, T., B. Tocque, I. Rey, M. Chassignol, N. T. Thuong, and C. Helene. Short modified antisense oligonucleotides directed against Ha-*ras* point mutation induce selective cleavage of the mRNA and inhibit T24 cell proliferation. *EMBO J.* **10**:1111–1118 (1991).
41. Chin, D. J., G. A. Green, G. Zon, F. C. Szoka, and R. M. Straubinger. Rapid nuclear accumulation of injected oligodeoxyribonucleotides. *New Biologist* **2**:1091–1100 (1990).
42. Leonetti, J. P., N. Mechti, G. Degols, C. Gagnor, and B. Lebleu. Intracellular distribution of microinjected antisense oligonucleotides. *Proc. Natl. Acad. Sci. USA* **88**:2702–2706 (1991).
43. Busen, W. Purification, subunit structure, and serological analysis of calf thymus ribonuclease HI. *J. Biol. Chem.* **255**:9434–9443 (1980).
44. Spector, D. Higher order nuclear organization: three-dimensional distribution

of small nuclear ribonucleoprotein particles. *Proc. Natl. Acad. Sci. USA* **87**:147–151 (1990).
45. Felgner, P. L., and G. M. Ringold. Cationic liposome-mediated transfection. *Nature* (*Lond.*) **337**:387–388 (1989).
46. Pontius, B. W., and P. Berg. Rapid renaturation of complementary DNA strands mediated by cationic detergents: a role for high-probability binding domains in enhancing the kinetics of molecular assembly processes. *Proc. Natl. Acad. Sci. USA* **88**:8237–8241 (1991).
47. Letsinger, R. L., G. Zhang, D. K. Sun, T. Ikeuchi, and P. S. Sarin. Cholesteryl-conjugated oligonucleotides: synthesis, properties, and activity as inhibitors of replication of human immunodeficiency virus in cell culture. *Proc. Natl. Acad. Sci. USA* **86**:6553–6556 (1989).
48. Kabanov, A. V., S. V. Vinogradov, A. V. Ovcharenko, A. V. Krivonos, N. S. Melik-Nubarov, V. I. Kiselev, and E. S. Severin. A new class of antivirals: antisense oligonucleotides combined with a hydrophobic substituent effectively inhibit influenza virus reproduction and synthesis of virus-specific proteins in MDCK cells. *FEBS Lett.* **259**:327–330 (1990).
49. Shea, R. G., J. C. Marsters, and N. Bischofberger. Synthesis, hybridization properties and antiviral activity of lipid-oligodeoxynucleotide conjugates. *Nucleic Acids Res.* **18**:3777–3783 (1990).
50. Stevenson, M., and P. L. Iverson. Inhibition of human immunodeficiency virus type 1-mediated cytopathic effects by poly(L-lysine)-conjugated synthetic antisense oligonucleotides. *J. Gen. Virol.* **70**:2673–2682 (1989).
51. Degols, G., J.-P. Leonetti, N. Mechti, and B. Lebleu. Antiproliferative effects of antisense oligonucleotides directed to the RNA of c-*myc* oncogene. *Nucleic Acids Res.* **19**:945–948 (1991).
52. Stein, C. A., R. Pal, A. L. DeVico, G. Hoke, S. Mumbauer, O. Kinstler, M. G. Sarngadharan, and R. L. Letsinger. Mode of action of 5′-linked cholesteryl phosphorothioate oligodeoxynucleotides in inhibiting syncyia formation and infection by HIV-1 and HIV-2 *in vitro*. *Biochemistry* **30**:2439–2444 (1991).
53. Loke, S. L., C. Stein, X. Zhang, M. Avigan, J. Cohen, and L. M. Neckers. Delivery of c-*myc* antisense phosphorothioate oligodeoxynucleotides to hematopoietic cells in culture by liposome fusion: specific reduction in c-*myc* protein expression correlates with inhibition of cell growth and DNA synthesis. *Curr. Top. Microbiol. Immunol.* **141**:282–289 (1988).
54. Behr, J.-P., B. Demeneix, J.-P. Loeffler, and J. Perez-Mutul. Efficient gene transfer into mammalian primary endocrine cells with lipopolyamine-coated DNA. *Proc. Natl. Acad. Sci. USA* **86**:6982–6986 (1989).
55. Leventis, R., and J. R. Silvius. Interactions of mammalian cells with lipid dispersions containing novel metabolizable cationic amphiphiles. *Biochim. Biophys. Acta* **1023**:124–132 (1990).

Send reprint requests to: C. Frank Bennett, Department of Molecular and Cellular Biology, ISIS Pharmaceuticals, 2280 Faraday Ave., Carlsbad, CA 92008.