Chemical modification of antisense oligonucleotides to increase nuclease resistance may improve their efficacy within enzyme-rich cellular targets (e.g. macrophages). We evaluated a panel of morpholino antisense oligomers (M-AS) for their ability to inhibit macrophage tumor necrosis factor-α (TNF-α) release and compared them to phosphodiester (O-AS) and phosphorothioate (S-AS) types of oligonucleotides. M-AS inhibited translation in vitro (rabbit reticulocyte lysate) of target mRNA at concentrations as low as 200 nM (e.g. percent inhibition by M-AS 2 at 0.2, 1.0, and 2.0 μM was 40.9 ± 5.3%, 50.2 ± 4.6%, and 57.7 ± 3.6% respectively, n = 4, p = 0.002 versus control). Similarly, M-AS 2 effectively, albeit partially, inhibited TNF-α production by LPS-stimulated macrophages (RAW 264.7 cells). Incubation of cells with 25 μM M-AS 2 resulted in 32.6 ± 2.6% (n = 3, p = 0.002 versus control) decrease in TNF-α release. In contrast, S-AS inhibited translation of the target mRNA in the rabbit reticulocyte lysate assay, but not in the cell-based assay. In fact, S-AS nonspecifically augmented TNF-α release. O-AS were without effect in either system. Uptake studies with fluorescent M-AS revealed that inhibitory effects were seen despite relatively low cellular uptake (intracellular concentration 30.5 ± 6.7 nm; efficiency of uptake 0.1%). In contrast, flow cytometric and confocal analysis revealed that S-AS were avidly taken up by RAW 264.7 cells, confirming that their lack of efficacy was not due to lack of uptake. With improved methods of delivery, M-AS may represent an important therapeutic modality.

Antisense oligonucleotides offer the potential for sequence-specific inhibition of gene expression in vitro and in vivo (for reviews, see Refs. 1–3). Initial antisense experiments employed phosphodiester oligonucleotides to inhibit gene expression; however, such oligonucleotides are susceptible to degradation by exon- and endonucleases, which are ubiquitous in serum and in the intracellular milieu (1, 3, 4). Chemical modification of oligonucleotides to enhance their stability is one approach to overcome this obstacle. Modifications that impart resistance to nuclease degradation include phosphorothioate, methylphosphonate, and 2-O-methyl derivatives (5). Phosphorothioate oligonucleotides are commonly used and effectively inhibit synthesis of proteins in various cell types. Due to their negative charge, however, phosphorothioate oligonucleotides interact with a number of cellular and viral proteins and have been associated with a number of nonspecific effects. Examples of these “sequence-independent” effects include induction of viral replication (6) and DNA polymerases (7) and activation of the transcription factor Sp1 (8).

Another strategy for chemical modification is to alter the ribose moiety. Morpholino oligomers are uncharged molecules in which the ribose moiety has been converted to a morpholino group (9) (Fig. 1). Early studies have found that these oligomers are highly specific and resistant to nuclease degradation (10). We now report a detailed investigation of morpholino-type antisense oligomers, using tumor necrosis factor-α (TNF-α)3 as the target mRNA. TNF-α is a pro-inflammatory cytokine, which mediates a number of immune and inflammatory responses (11) and is produced by a number of cell types, including macrophages, mast cells, and T-lymphocytes. TNF-α is released early in the inflammatory cascade and triggers a wide range of cellular events (12) including induction of interleukin-1, interleukin-8, and platelet-activating factor production, up-regulation of the expression of adhesion molecules on neutrophils and endothelial cells, enhancement of vascular permeability (11, 13, 14), and stimulation of neutrophil respiratory burst activity (15). Importantly, inhibition of TNF-α by neutralizing antibodies and/or soluble TNF-α receptor abrogates many deleterious effects in several models of inflammation (14, 16–19). Hence, antisense oligonucleotides that successfully target TNF-α may also prove useful as anti-inflammatory agents.

We evaluated morpholino oligomers, as well as phosphodiester and phosphorothioate oligonucleotides for their ability to inhibit TNF-α expression in two ways. First, the efficacy of the oligos was assessed by quantitating their inhibition of in vitro translation of a TNF-α-luciferase reporter mRNA. Subsequently, we measured the ability of antisense agents to inhibit lipopolysaccharide (LPS)-induced TNF-α production in a mouse macrophage-like cell line (RAW 264.7). We found that morpholino-type oligomers significantly inhibited TNF-α production in a sequence-specific manner, suggesting potential utility of this modification in the design of therapeutic agents.

MATERIALS AND METHODS

Oligonucleotides—Morpholino oligonucleotides were modified and coumarin-linked were provided by AntiVirals, Inc., and were prepared by methods described previously (9, 20). Phosphodiester, phosphorothioate, and fluorescein (FITC)-conjugated phosphorothioate oligonucleotides were

1 The abbreviations used are: TNF-α, tumor necrosis factor-α; oligo, oligonucleotide; LPS, lipopolysaccharide; FITC, fluorescein isothiocyanate; PCR, polymerase chain reaction; RRL, rabbit reticulocyte lysate; PAGE, polyacrylamide gel electrophoresis; PBS, fetal bovine serum; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay.
prepared on a Cruachem PS250 synthesizer using standard phosphoramidite methods.

Preparation of Plasmid Constructs—Murine tumor necrosis factor-α (TNF-α) cDNA in pBR322 (21) was obtained from ATCC (American Type Culture Collection, Rockville, MD). A reporter plasmid encoding luciferase enzyme (pT3/T7-LUC) was obtained from Clontech (Palo Alto, CA). The 5′ untranslated region and the first 38 coding bases of TNF-α were amplified by pBR322-TNF-α polymerase chain reaction (PCR) using specific primers. The primers were designed to incorporate a PstI site at the 5′ end and an XbaI site at the 3′ end of the PCR product. The pT3/T7 plasmid and PCR product were digested with PstI and XbaI, and the PCR product was inserted at the 5′ end of the luciferase coding region (bases 47–1900). Sequence analysis (using Sequenase version 2.0, U. S. Biochemical Corp.) confirmed that the 5′ region of TNF-α is inserted in frame with the luciferase coding region.

In Vitro Transcription—Messenger RNA (mRNA) was transcribed to the plasmid construct described above using Megascript T3 and T7 polymerase kits (Ambion, Inc., Woodward, TX) according to the specifications of the manufacturer. Briefly, 1 μg of cDNA, 7.5 μm ribonucleotide triphosphates (ATP, CTP, GTP, UTP), transcription buffer, and enzyme mix were combined in a total volume of 20 μl. The mixture was incubated for 4 h at 37 °C. After incubation the DNA template was digested with RNase-free DNase I. The reaction was quenched by the addition of sodium acetate (NaOAc), and RNA was purified by phenol-chloroform extraction and isopropanol precipitation. The resolution of a single transcript size of 1.9 kilobases upon ethidium bromide staining of a denaturing agarose gel confirmed the quality of these transcripts.

In Vitro Translation—In vitro translation reactions were performed using a nuclease-treatment rabbit reticulocyte lysate (RRL) kit from Promega Corp. (Madison, WI). To allow annealing of the antisense agent and the mRNA, a preincubation was performed as described below. Preincubation mixtures contained 1.0 μl (1.0 μg) of mRNA template, 1.0 μl of antisense solution, and 4.0 μl of diethylpyrocarbonate-treated water; control preincubations contained 1 μl of RNA template and 5.0 μl of diethylpyrocarbonate-treated water. Preincubation mixtures were heated to 70 °C for 5 min and slowly cooled to 37 °C. Upon cooling, 0.1 μl of 1 μM amino acid mixture deficient in methionine, 0.5 μl of mRNA (40 units/μl, Promega Corp.), 0.5 μl of [35S]methionine (>1000 Ci/mmol, DuPont NEN, Boston, MA), and 17.5 μl of reticulocyte lysate were added to the preincubation mixtures. Reactions were incubated at 30 °C for 60 min and stopped by chilling to 4 °C. The results of the translation reaction were assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography.

Measurement of Luciferase Activity—Luciferase synthesized during in vitro translation was quantitated by assay of enzyme-dependent light production using a commercial kit (Promega Corp.). Ten μl of each sample was placed in a 5-ml polystyrene test tube and the tubes were then placed in G. B. Berthold luminometer (G. B. Berthold, Gaithersburg, MD). At the time of measurement, 100 μl of luciferase substrate was automatically injected into each sample, and total luminescence was measured over a 20-s time interval.

SDS-PAGE and Analysis—Reticulocyte lysate reactions were also analyzed by SDS-PAGE and autoradiography. Acrylamide gels (12%) were loaded with 2 μl of each translation reaction, subjected to constant voltage (80 V) for 1.5–2 h, then fixed for 15 min in 40% methanol/10% acetic acid and dried under vacuum. Gels were exposed to Kodak Biomax MR scientific imaging film (Eastman Kodak Co.). Prestained molecular weight markers (Bio-Rad) were run on each gel to allow for estimation of molecular weight. Relative protein levels were quantitated by densitometric analysis using a Scan II densitometer (Hewlett-Packard, Palo Alto, CA) and NIH Image (version 1.62) software.

Cell and Culture Reagents—RPMI 1640 medium, L-glutamine, penicillin, and streptomycin were purchased from Sigma. Fetal bovine serum (FBS) was purchased from HyClone (Logan, UT). RAW 264.7 cells, a murine macrophage line, were obtained from ATCC. Cells were cultured in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. The cells were maintained at 37 °C with 5% CO2 in a humidified incubator.

Treatment of Cells with Antisense Agents—Two days prior to use in the assay, cells were seeded at 6 × 105 in 20 ml in a 75 cm2 tissue culture plate (Corning-Costar, Cambridge, MA). One day before the assay, cells were harvested by treatment with phosphate-buffered saline (PBS)-EDTA and plated in 96-well format at 2 × 104 cells/well (100 μl volume) in RPMI-0.5% FBS. On the day of treatment, antisense agents at indicated doses were mixed with Lipofectin (10 μg/ml) (Life Technologies Inc.) in serum-free RPMI for 15 min at room temperature. Lipofectin has been shown to improve the uptake and efficacy of phosphorothiate and phosphodiester oligonucleotides in other systems (22, 23). The oligo-Lipofectin mixtures were added to the RAW 264.7 cells and were incubated at 37 °C for 4 or 6 h. After this preincubation, cells were stimulated with 37.5 ng/ml lipopolysaccharide (LPS, Sigma) and incubated for an additional 4 h in a final concentration of 0.5% FBS. Preliminary dose-response analysis indicated that this concentration of LPS caused approximately 60% of the maximum TNF-α response. Specifically, treatment with 37.5 ng/ml LPS resulted in production of 1.12 ± 0.12 ng/ml TNF-α compared with 1.7 ± 0.06 ng/ml at 1 μg/ml LPS (at plateau). Upon completion of the incubation period, cell supernatants were harvested and their TNF-α protein content was measured with the aid of RAW 264.7 cells with Lipofectin. The production of TNF-α was quantitated by the addition of antisense solution, and 4.0 μl of diethylpyrocarbonate-treated water. Preincubation mixtures were added to the preincubation mixtures. Reactions were incubated at 30 °C for 60 min and stopped by chilling to 4 °C. The results of the translation reaction were assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography.

Measurement of Uptake of Fluorescent Oligo—One day before treatment, RAW 264.7 cells were plated in 24-well format with 1 × 106 cells/well in 500 μl. On the day of treatment, RAW 264.7 cells were incubated with coumarin-labeled morpholino oligomers, M-AS 2 and M-AS 3, in the presence of Lipofectin (10 μg/ml) for 4 h. Following incubation, the cells were harvested, washed twice with PBS, and the amount of cell-associated morpholino oligonucleotide was determined by flow cytometry and by fluorescence (excitation 373, emission 457) of the total cell population. The concentration of oligo associated with RAW 264.7 cells was estimated by interpolation from a standard curve of known coumarin (Molecular Probes, Eugene, OR, catalog no. D-126). The presence of intracellular oligomer was assessed by fluorescence confocal microscopy.

Using the same protocol described above, the uptake of FITC-labeled oligonucleotides was determined. RAW 264.7 cells were incubated with FITC-labeled phosphorothiate oligonucleotide (12.5 or 125 μM) in the presence of Lipofectin (10 μg/ml) for 4 or 6 h. Following incubation, cells were harvested and washed twice with PBS, cytocentrifuged (200 × g), and the amount of cell-associated oligonucleotide was determined using flow cytometry. Intracellular localization of FITC-labeled oligonucleotide was confirmed by fluorescent confocal microscopy.

Statistical Analysis of Data—Statistical significance of mean comparisons was determined by analysis of variance with Scheffe’s post hoc test to control for multiple comparisons using Statview statistical software package (Abacus Concepts, Inc., Berkeley, CA).

RESULTS

Effect of Antisense on Translation of Target mRNA in a Cell-free System—To compare the relative efficacy of phosphorothiate, phosphodiester, and morpholino antisense oligomers, we evaluated their ability to block translation of a target mRNA in a cell-free system (rabbit reticulocyte lysate). Five morpholino oligomers (M-AS) were evaluated in this system. Four of the oligomers were complimentary to sequences within the 5′-untranslated region or the AUG (initiation site) region of TNF-α (Fig. 2). These regions of mRNA have been successfully targeted by antisense oligonucleotides in other experimental systems (for review see Ref. 24). The fifth morpholino oligomer (M-NS) was selected randomly, was not complementary to TNF-α, and was used to control for nonspecific effects.

TNF-luciferase mRNA served as the target sequence in this
system. This construct is a hybrid sequence which contains the 5'-untranslated region and the first 38 coding bases of TNF-\(\alpha\) mRNA targeted by antisense. Numbered bars below the sequence indicate the regions targeted by the antisense oligos. B, the antisense sequences used in this study are described. Antisense agents are designated according to the type (M = morpholino, S = phosphorothioate, O = phosphodiester), and whether they are antisense (AS) or nonsense (NS). The site targeted by each oligo is outlined above. Oligos that share numerical designation have the same target site.

Efficacy of Morpholino-modified Antisense Oligomers

Table I shows that M-AS on TNF-luciferase mRNA in vitro protein production in samples incubated in the presence of [\(\gamma\)-\(\text{S}\)]methionine and analyzed by SDS-PAGE/autoradiography. A representative autoradiograph shows abundant protein production in the absence of antisense oligomers. Translation was performed in the presence of [\(\gamma\)-\(\text{S}\)]methionine and analyzed by SDS-PAGE/autoradiography. A representative autoradiograph shows abundant protein production in the absence of antisense oligomers.

Table: Morpholino oligomers inhibit translation of TNF-luciferase mRNA in vitro

| Oligomer | % of control light production (± S.E.) |
|----------|-----------------------------------------|
|          | 0.2 \(\mu\)M | 1.0 \(\mu\)M | 2.0 \(\mu\)M |
| M-AS 1   | 38.9 ± 1.9 | 19.2 ± 2.1 | 21.5 ± 1.1 |
| M-AS 2   | 32.8 ± 1.7 | 14.2 ± 1.6 | 9.3 ± 2.4 |
| M-AS 3   | 45.1 ± 4.7 | 21.0 ± 1.7 | 23.4 ± 3.0 |
| M-AS 4   | 37.7 ± 4.2 | 8.3 ± 3.2  | 8.7 ± 2.9  |
| M-NS     | 91.2 ± 1.0 | 81.8 ± 4.7 | 89.6 ± 4.1 |

\(\text{M}, \text{M-AS} 2 \text{and M-AS} 4 \text{caused a significant reduction (52.5\% and 76.2\%, p = 0.0024 and p < 0.0001, respectively) in translation compared to the control, while M-AS 3 did not significantly inhibit translation. Similar results were obtained at 0.2 \(\mu\)M. The nonsense-M oligomer had no effect on translation at any of the concentrations tested. Notably, the effects of M-AS 1-4 measured in the \(\gamma\)-\(\text{S}\) incorporation assay were consistent with those found in the light production assay.}

We next sought to directly compare the efficacy of morpholino oligomers and phosphodiester and phosphorothioate oligonucleotides. Phosphodiester and phosphorothioate oligonucleotides were evaluated for their ability to inhibit translation of the target mRNA in a sequence-specific manner. Specifically, three phosphorothioate oligonucleotides (S-AS) were evaluated for their ability to inhibit translation of the TNF-luciferase construct in vitro. Two

\[
\frac{\text{Untranslated region}}{\text{Control mRNA sequence}}
\]

\[
\frac{\text{Control mRNA}}{\text{Antisense oligos}}
\]

\[
\frac{\text{Antisense oligos}}{\text{Control mRNA}}
\]
oligonucleotides caused comparable sequence-independent (nonspecific) effect. Both specific (S-AS 2 and S-AS 4) and control (S-NS) phosphorothioate gomers caused sequence-specific inhibition of translation at all concentrations of the oligonucleotides were complimentary to TNF-α.

In our cell-free system.

We next tested the ability of the same five (M-AS 2, M-AS 4, and M-NS) showed nonspecific effects at 2 μM and no effect at 1 or 0.2 μM. The amount of protein production was determined by densitometric analysis of samples subjected to SDS-PAGE/autoradiography. Bars indicate the mean percentage of control translation ± S.E., n = 4. Control samples run in the absence of oligo were used to establish 100%. * indicates statistical significance, p < 0.01.

Efficacy of Morpholino-modified Antisense Oligomers

of the oligonucleotides were complimentary to TNF-α (S-AS 2 and S-AS 4), and one was a nonsense control (S-NS). The base sequence of these oligonucleotides was matched to the morpholino oligomers M-AS 2, M-AS 4, and M-NS, respectively. At 2.0 and 1.0 μM, S-AS 2 and S-AS 4 significantly inhibited translation of TNF-luciferase; however, the nonspecific control (S-NS) also significantly inhibited translation (Fig. 4B). No effect of the S-AS on translation was apparent at a concentration of 0.2 μM. Hence, these phosphorothioate oligonucleotides did not exhibit specific, sequence-dependent inhibition of translation in our cell-free system.

Three phosphodiester oligonucleotides (O-AS) were also assessed at concentrations of 2.0, 1.0, and 0.2 μM (Fig. 4C). The sequences of the O-AS were matched to those of the S-AS and M-AS. O-AS 2 and O-AS 4 at a concentration of 2 μM significantly inhibited (32.5% and 28.2%, p = 0.0033 and 0.0095, respectively) translation of TNF-luciferase compared to the control; however, this was not statistically different from the nonspecific effect of O-NS (p > 0.26). None of the phosphodiester oligonucleotides caused significant inhibition of translation at concentrations of 1.0 and 0.2 μM. Thus, in contrast to their morpholino counterparts, these phosphodiester oligonucleotides were not effective inhibitors of translation in our cell-free system.

FIG. 5. Dose response analysis of inhibition of LPS-induced TNF-α production by morpholino oligomer M-AS 2. RAW 264.7 cells were preincubated with either M-AS 2 (white bars) or M-NS (black bars) (25 μM, 12.5 μM, or 6.25 μM) for 6 h in the presence of 10 μg/ml Lipofectin, then stimulated with 37.5 ng/ml LPS, and after 4 h TNF-α in cell supernatants was quantitated by ELISA. Results shown represent the mean percent inhibition of TNF-α production ± S.E., n ≥ 5 experiments performed in triplicate. TNF-α produced in samples treated with Lipofectin only was used to establish the 0% inhibition line. * indicates statistically significant inhibition, p < 0.001.

The efficacy of morpholino oligomers (A) and phosphorothioate (B) and phosphodiester (C) oligonucleotides at concentrations of 2.0, 1.0, and 0.2 μM was compared using in a cell-free system (RRL). Morpholino oligomers caused sequence-specific inhibition of translation at all concentrations tested (M-AS 2 and 4 > M-AS 1 and 3), while M-NS had no effect. Both specific (S-AS 2 and 4) and control (S-NS) phosphorothioate oligonucleotides caused comparable sequence-independent (nonspecific) inhibition at 1 and 2 μM (see "Discussion"). Phosphodiester oligonucleotides (O-AS 2, O-AS 4, and O-NS) showed nonspecific effects at 2 μM and no effect at 1 or 0.2 μM. The amount of protein production was determined by densitometric analysis of samples subjected to SDS-PAGE/autoradiography. Bars indicate the mean percentage of control translation ± S.E., n = 4. Control samples run in the absence of oligo were used to establish 100%. * indicates statistical significance, p < 0.01.

Effect of Antisense on TNF Production by LPS-stimulated Macrophages—We next tested the ability of the same five M-AS described above to inhibit TNF-α production by RAW 264.7 cells. Cells were preincubated with antisense at three concentrations (25, 12.5, and 6.25 μM) for 4 or 6 h prior to stimulation with LPS (37.5 ng/ml). The results demonstrate that only the oligomer designated M-AS 2 significantly inhibited TNF-α production by RAW 264.7 cells (Fig. 5). At 25 μM, oligomer M-AS 2 achieved significant inhibition of TNF-α production with both 4 and 6 h preincubation (29.4 ± 4.9%, n = 6, p = 0.004; and 32.6 ± 2.6% inhibition, n = 6, p = 0.0008, respectively). Dose-response analysis revealed that extending the preincubation time to 6 h slightly improved the inhibition achieved and reduced the variability observed. For example, inhibition by 12.5 μM M-AS 2 was statistically significant after 6 h of preincubation (29.2 ± 7.7% inhibition, n = 6, p = 0.044) but was more variable and not significant after 4 h of preincubation (20.3 ± 11.5% inhibition, n = 3, not shown). Also, with 6 h of preincubation inhibitory effects of oligomer M-AS 2 were still evident at 6.3 μM (12.9 ± 5.2% inhibition, n = 6), while no effect of 6.3 μM M-AS 2 was observed with 4 h of preincubation. Lower concentrations of M-AS 2 (3.1, 1.6, and 0.8 μM) did not inhibit TNF-α production with either 6 or 4 h of preincubation (not shown).

Trypan blue staining showed that the viability of cells treated with M-AS and M-NS was equal (≥85%, data not shown), demonstrating that the inhibition of TNF-α production achieved by M-AS 2 was not due to cytotoxicity.

Treatment with 25 μM of the remaining three morpholino oligomers (M-AS 1, M-AS 3, and M-AS 4) for 4 or 6 h caused slight but not significant inhibition of TNF-α production by RAW 264.7 cells (Fig. 6). Treatment with 12.5 or 6.3 μM of these oligomers did not inhibit TNF-α production at either 4 or 6 h. The nonsense control oligomer caused no inhibition under any of the parameters examined.

One factor that could potentially limit the efficacy of antisense treatment is the presence of a preformed pool of the target protein. To determine the contribution of preformed
performed in triplicate. TNF-α production by RAW 264.7 cells was assessed using FITC-labeled phosphorothioate (S), and phosphodiester (O) types. The results obtained using M-AS indicated that oligomer sequence M-AS2 was the most effective inhibitor of cellular TNF-α production.

In light of this finding, S and O oligonucleotides that bind to the same mRNA site as M-AS 2 were synthesized and evaluated in the RAW 264.7 cell model. A nonsense S oligonucleotide was also included as a control in this study. The S and O oligonucleotides were evaluated after 4 and 6 h of preincubation with Lipofectin for 4 h. The amount of cell-associated oligomer was determined by both fluorometric and flow cytometric analysis. These two methods demonstrated that the treated cells exhibit a 2-fold increase in fluorescence over the controls (Table III, Fig. 7). Histograms of the flow cytometry data indicate a shift in the whole cell population rather than increased fluorescence within a subpopulation. Fluorometry was used to estimate the amount of coumarin-linked oligomer taken up by RAW 264.7 cells by comparison with a standard curve of coumarin. The efficiency of uptake was poor; with intracellular concentrations of oligomer of 30.5 ± 6.7 nM, representing uptake of 0.1% of the total available (25 μM) oligomer. The relative uptake of M-AS 2 and M-NS was equal, ruling out the possibility that the inhibitory effects of M-AS 2 were due to preferential uptake compared to the nonsense (M-NS). Confocal microscopy also confirmed the presence of intracellular oligomer (not shown).

Similarly, the uptake of FITC-labeled phosphorothioate oligonucleotides was determined by flow cytometry. RAW 264.7 cells were treated with either 12.5 or 1.25 μM oligonucleotide in the presence of Lipofectin for 4 or 6 h. A representative histogram shown in Fig. 8A illustrates a broad distribution of fluorescence among the cells of this population, without discrete subpopulations. The results demonstrate that greater than 95% of the cell population treated with fluorescent oligonucleotide displayed increased fluorescence compared with the control cells (Fig. 8B). Confocal microscopy demonstrated intracellular localization of the phosphorothioate oligonucleotide, primarily as discrete foci of fluorescence suggestive of vesicular structures. This confirms that the failure of phosphorothioate oligonucleotides to inhibit TNF-α production by RAW 264.7 cells is not due to an inability to take up the oligonucleotides; however, it suggests that these oligonucleotides are delivered to inappropriate cellular compartments.

**DISCUSSION**

Chemical modifications of antisense oligonucleotides may improve their ability to specifically inhibit gene expression within nuclease-rich cellular environments. In this study we compared morpholino-modified oligomers to the more commonly used phosphodiester and phosphorothioate oligonucleotides. We first measured their ability to inhibit translation of a TNF-α luciferase reporter construct in vitro. We then tested their effects on TNF-α production by a macrophage-like cell line (RAW 264.7). In both systems, we found that morpholino oligomers significantly inhibited gene expression in a sequence-
dependent manner, while no sequence-specific inhibition was observed with phosphodiester and phosphorothioate oligonucleotides.

We postulated that the in vitro translation system might serve as a screening assay to demonstrate that the antisense sequences selected recognize and are effective against the TNF-α mRNA. A plasmid construct was made, which contained the 5'-untranslated region and the first 38 coding bases of TNF-α spliced with the coding region of luciferase, a useful reporter molecule (25) whose activity is easily measured by a highly sensitive light production assay. In this study, the light production assay demonstrated significant and specific inhibition of luciferase production by morpholino oligomers; however, the use of this assay for direct comparison of the three types of antisense was precluded by the fact that phosphorothioate oligonucleotides were found to directly inhibit luciferase activity in a nonspecific manner. Instead, the reporter construct was used in the in vitro translation experiments and the efficacy of oligos was determined by densitometric analysis after SDS-PAGE/autoradiography. Under in vitro translation conditions, the morpholino oligomers tested proved to be highly efficient and specific inhibitors of their target mRNA. These oligos showed effects at 0.2 μM, which represents an oligo to RNA ratio of 0.8:1. This ratio is lower than what has been reported to be necessary to achieve inhibition by phosphorothioate oligonucleotides (e.g. 10:1 for oligonucleotides >20 bases in length and 50:1 for oligonucleotides 10–15 bases) (26). The effects of phosphodiester and phosphorothioate oligonucleotides at all concentrations tested were largely nonspecific (i.e. sequence-independent). Thus, we conclude that such a system may be somewhat useful as a screening procedure for morpholino oligo-

### Table III

Fluorometric analysis of uptake of coumarin-labeled morpholino oligomers

| Oligomer | Mean fluorescence (± S.E.) | Estimated intracellular concentration nM |
|----------|-----------------------------|------------------------------------------|
| M-AS 2   | 14.8 ± 4.1                  | 28.7 ± 6.1                               |
| M-NS     | 14.5 ± 0.8                  | 29.7 ± 4.5                               |
| None     | 7.2 ± 0.8                   |                                          |

**Fig. 8. Flow cytometric analysis of FITC-labeled phosphorothioate oligonucleotide uptake by RAW 264.7 cells.** Representative histograms of RAW 264.7 cells incubated with 12.5 μM FITC-labeled phosphorothioate oligonucleotide S-AS 2 in the presence of Lipofectin (A) or control cells incubated with Lipofectin alone (B) are shown. Greater than 95% of cells incubated with FITC-labeled oligonucleotide exhibited positive (> 2 x control) fluorescence. The histogram reveals that the population of positive cells was broad and without discrete subpopulations. (Mean fluorescence after treatment of cells with 12.5 or 1.25 μM S-AS 2 or Lipofectin alone was 233.9 ± 91, 131.5 ± 35.5, and 1.3 ± 0.4, respectively; n = 4.)
gomers; however, luciferase-based assays are not useful for screening potentially effective phosphorothioate oligonucleotides because of substantial nonspecific effects.

We also investigated the ability of the antisense agents to inhibit TNF-α production by a macrophage-like cell line (RAW 264.7) in response to a biological stimulus (LPS). The morpholino oligomers tested were able to inhibit LPS-stimulated TNF-α production by RAW 264.7 cells in a sequence-specific and dose-dependent manner. We found that the magnitude of inhibition achieved by morpholino oligomers varied among the sequences tested. The superior efficacy of M-AS 2 and M-AS 1 and 3 was consistent with results observed in the RRL assay. In contrast, M-AS 4 was less effective than predicted by the RRL. This is in agreement with other reports that an effective antisense oligonucleotide for any target sequence must be determined empirically. In contrast with the morpholino oligomers, the phosphorothioate and phosphodiester oligonucleotides tested failed to inhibit (in fact, augmented) TNF-α production.

The augmentation of TNF-α production by phosphorothioate oligonucleotide is consistent with a substantial literature describing the nonspecific effects of phosphorothioate oligonucleotides. These sequence-independent effects include inhibition of DNA polymerases, inhibition of viral replication, and activation of the transcription factor Sp1 (6–8). The TNF-α promoter does contain an Sp1 binding motif; however, the effect of this transcription factor on TNF-α expression remains unclear. A study by Ziegler-Heitbrock et al. (27) indicates that in LPS-stimulated human monocytes NF-κB, not Sp1, is essential for the induction of TNF-α expression. Similar findings have been reported in murine cells (28, 29). Perhaps the activation of Sp1 by phosphorothioate oligonucleotides primes the cells for an enhanced response to LPS stimulus.

The uptake of coumarin-labeled morpholino oligomers was evaluated by flow cytometry, fluorometry, and confocal microscopy. These studies identified an important limitation to the efficacy of morpholino oligomers within the macrophage cell targets. The data indicate that the uptake of morpholino oligos was low (intracellular concentrations 30–67 nM, 0.1% efficiency of uptake). Despite this poor uptake, significant inhibition of TNF-α production was achieved by these oligomers. The 32% inhibition of TNF-α observed at these intracellular concentrations is consistent with the results obtained in the RRL system (i.e., 40% inhibition at 200 nM). The uptake of FITC-labeled phosphorothioate oligonucleotides was similarly assessed by flow cytometry and confocal microscopy. There was substantial intracellular uptake of the oligonucleotides at both the 4- and 6-h time points. Thus, we conclude that the failure of phosphorothioate oligonucleotides to inhibit TNF-α production is not attributable to lack of uptake. The nonspecific augmentation of TNF-α production also supports this conclusion. Delivery of the phosphorothioate and phosphodiester oligonucleotides to inappropriate cellular compartments (e.g., phagolysosomes) or degradation of these oligonucleotides within the cell may also contribute to their lack of inhibitory effects.

An established limitation of antisense therapy is delivery of adequate amounts to relevant intracellular compartments. To enhance the uptake of phosphodiester and phosphorothioate oligonucleotides, we used Lipofectin, a commercially available cationic lipid preparation. The cationic lipid is thought to form complexes with the negatively charged oligonucleotides. Those complexes are then internalized by cells. Since morpholino oligomers are uncharged, no a priori rationale for improved delivery by Lipofectin exists. We included this agent with the morpholino oligomers to allow a fair comparison of the efficacy of morpholino oligomers to phosphodiester and phosphorothioate oligonucleotides whose uptake and efficacy has been successfully improved by Lipofectin (22, 23). Comparison experiments showed that Lipofectin did not affect maximal efficacy of morpholino oligomers in our cell culture system; however, it did diminish variability within triplicates tested. As mentioned above, despite the presence of Lipofectin, the efficiency of uptake of the uncharged morpholino oligomers is quite low (0.1%) compared with values that have been reported for uptake of phosphorothioate oligonucleotides (between 1 and 11%) (30).

The level of inhibition achieved in our experiments demonstrates the potential utility of morpholino oligomers. In order for such agents to be more useful therapeutics, however, a substantially higher level of inhibition will be required. Macrophages, the main source of TNF-α, are especially difficult targets for intracellular therapeutics because of their abundant degradative enzymes and their extensive phagocytic-endolysosomal pathways. It is likely that the efficacy of morpholino oligomers and other antisense agents will vary according to cell type. To test this hypothesis, the same oligos could be studied in a different cell type with a different cytoplasmic environment (i.e., not rich in degradative enzymes). Indeed, preliminary data indicate that the phosphorothioate oligonucleotides directed against TNF-α, which were ineffective in RAW 264.7 cells, are effective within T-cell targets.2

In summary, morpholino oligomers were effective sequence-specific inhibitors of in vitro mRNA translation. The morpholino oligomer M-AS 2 also caused significant inhibition of TNF-α production by macrophages despite remarkably low (nM) intracellular concentrations. With improved methods of delivery, morpholino oligos may represent important therapeutic agents within macrophages and other cells.

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