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Specific Inhibition of Macrophage TNF-α Expression by In Vivo Ribozyme Treatment

Kevin O. Kisich, Robert W. Malone, Paul A. Feldstein, and Kent L. Erickson

The overproduction of the cytokine TNF-α is associated with inflammatory and autoimmune diseases. We have developed a means to block TNF-α production with ribozymes directed against TNF-α mRNA to selectively inhibit its production in vitro and in vivo. Following cationic lipid-mediated delivery to peritoneal murine macrophages in culture, anti-TNF-α ribozymes were more effective inhibitors of TNF-α secretion than catalytically inactive ribozyme controls. Inhibition of TNF-α secretion was proportional to the concentration of ribozyme administered, with an IC_{50} of ~10 nM. After i.p. injection of cationic lipid/ribozyme complexes, elicited macrophages accumulated ~6% of the administered ribozyme. The catalytically active ribozyme suppressed LPS-stimulated TNF-α secretion by ~50% relative to an inactive ribozyme control without inhibiting secretion of another proinflammatory cytokine produced by macrophages, IL-1α. Ribozyme-specific TNF-α mRNA degradation products were found among the mRNA extracted from macrophages following in vivo ribozyme treatment and ex vivo stimulation. Thus, catalytic ribozymes can accumulate in appropriate target cells in vivo; once in the target cell, ribozymes can be potent inhibitors of specific gene expression. The Journal of Immunology, 1999, 163: 2008–2016.

Umor necrosis factor-α is a proinflammatory cytokine produced primarily by activated cells of the monocyte/macrophage lineage (1). Although TNF-α is necessary for normal immune responses to pathogens, when overproduced, TNF-α can cause acute shock-like symptoms (2), cachexia (3), or chronic autoimmune reactions such as rheumatoid arthritis or multiple sclerosis (4, 5). One method for blocking acute shock-like symptoms involves the use of Abs against TNF-α. For example, the development of sepsis in mice following LPS administration was blocked by pretreatment of the mice with anti-TNF-α neutralizing antiserum (6). Moreover, treatment of mice or humans with anti-TNF-α mAbs ameliorated many of the symptoms of rheumatoid arthritis (7).

Although TNF-α function can be blocked with soluble receptors for or mAb against TNF-α, we sought to develop a method to inhibit TNF-α production at the gene transcriptional level. Inhibition of TNF-α production may be a more efficient method in treating conditions associated with its overexpression than blocking protein binding, because no immune complexes would be formed. Thus, we have developed methods for synthesizing and delivering catalytic RNA specific for TNF-α and have used an in vivo murine peritoneal macrophage model to demonstrate and examine the biological response to the gene therapy.

One approach to block gene expression is through the use of ribozymes. Those RNA molecules have enzymatic properties to catalyze specific RNA cleavage. Thus, development of an effective ribozyme-based strategy to reduce gene expression requires the design and synthesis of oligonucleotides that efficiently cleave the target mRNA, as well as their delivery or expression within the appropriate cells. For that purpose, several ribozyme motifs including the group I intron of Tetrahymena thermophyla (8), self-splicing group II introns, hepatitis δ virus ribozymes (9), Neurospora VS RNA (10), Ribonuclease P, and hairpin and hammerhead ribozymes have been adapted to trans cleavage of substrate RNA (11, 12). Hammerhead motif ribozymes common to the (+) strands of plant satellite RNA have been shown to suppress gene expression in several experimental models (13–15). The hammerhead was chosen for these studies due to its requirements for a minimal substrate recognition sequence in the mRNA target, as well as its small size relative to other ribozyme motifs (11). Moreover, a number of the factors that may influence hammerhead ribozyme activity against TNF-α in vitro have been previously defined (16). Specificity of the ribozymes for TNF-α mRNA was achieved by altering the sequences in the 5′ and 3′ regions of the ribozymes such that they were complementary to the targeted region of TNF-α mRNA.

Ribozymes are not normally present in mammalian cells. Thus, to inhibit gene expression, ribozymes must enter the cytoplasm or nucleus where they can cleave the targeted mRNA. Cationic transfection lipids, cytofectins, are one group of vehicles used for in vitro delivery of RNA (17), including preformed ribozymes (13), into mammalian cells. RNA transfection into a promonocytic cell line has been demonstrated (13, 17), but reports documenting transfection of primary macrophages in vivo have yet to be published. Different cultured cells appear to require different cytofectins for optimal delivery of polynucleotides.

Ribozymes must be catalytically active after delivery to the target to offer advantages over antisense oligonucleotides. Previous reports of ribozyme inhibition of TNF-α secretion did not demonstrate this key feature. In this paper, we have demonstrated that...
ribozymes consisting of only RNA can be delivered efficiently and intact to the appropriate target cells in vivo. We have evaluated four cationic lipid formulations for ribozyme delivery, both in vitro and in vivo, by assessing the amount of intact ribozyme within the cells as well as the degree of inhibition of TNF-α. Hammerhead ribozymes delivered to macrophages in vivo were biologically active by virtue of their ability to inhibit TNF-α secretion and catalyze the degradation of the TNF-α mRNA into smaller fragments, activities that were dependent on the catalytic capacity of the ribozymes.

Materials and Methods

Ribozyme design and synthesis

Ribozymes were designed according to the method previously described (11, 16), with modifications. Ribozyme sites were chosen based on proximity to the beginning of the coding region of the mRNA (rz254 and rz254sp) or production of fragments of similar electrophoretic mobility (rz442 and rz442sp) (16). Rz254 was targeted to cleave at position 254 and rz442 was targeted to cleave at position 442 of the TNF-α cDNA. Rz254sp and rz442sp were self-processing (sp), whereas rz254 and rz442 had the self-processing domains removed. Mini monomer (mm) constructs, rzmm442/24 and rzmm442/10, were based on site 442 for comparison with the linear ribozymes rz442 and rz442sp. For that, rz42 was inserted into the variable domain of an autocatalyzing hairpin ribozyme, m10 (12) to create rzmm442/24. Reduction of the target recognition bases of the linear ribozymes rz442 and rz442sp was predicted to 0.1 mM to achieve the correct specific activity.

unlabeled ribozyme in some experiments to achieve the correct specific activity labeled ribozymes; it was purified by size exclusion chromatography with 1 mM for unlabeled CTP. This ratio resulted in high specific activity labeled ribozymes delivered to macrophages in vivo were biologically active by virtue of the catalytic capacity of the ribozymes.

Labeling of ribozymes

For studies of stability in tissue culture and in vivo biodistribution, ribozymes were all assayed at 40 nM, with a substrate concentration of 0.4 nM. RNA substrate for site 254 had the sequence 5'-GGTGCTATGTCCTGAGCTTCTTCT-3'; RNA substrate for site 442 had the sequence 5'-TGTAGCCCACGTGAGCAACAC-3'. Substrates were synthesized in vitro transcription of fully complementary DNA oligonucleotides. Substrates were 5'-end labeled by exchange phosphorylation with [γ-32P]ATP (NEN) and T4 polynucleotide kinase (Promega). 32P end-labeled substrate were purified by electrophoresis on 12% polyacrylamide gels containing 7 M urea, followed by excision and elution into 1 mM EDTA. Excess salts and urea were removed by column chromatography over Sephadex G-25 (Pharmacia) before addition of purified, unlabeled substrate to the correct specific activity. Ribozyme and 32P end-labeled substrate were diluted to a final concentration in 10 mM MgCl2, and 50 nM Tris (pH 7.5) and individually heated briefly to 90°C to disrupt secondary structure. Ribozyme and substrate were then cooled to 37°C and combined. Aliquots of the reaction mixture were removed at 0, 1, 5, 30, and 120 min and mixed with a stop buffer of 5 nM EDTA, 97% formamide, and 0.01% bromophenol blue at 4°C. The percentage of the substrate remaining intact was determined by gel electrophoresis of the reaction aliquots and quantitated by autoradiography with storage phosphor screens (PhosphorImager, Molecular Dynamics, Sunnyvale, CA). The fraction of substrate uncleaved vs time were fit to a double exponential function, y = m1e-a1t + m2e-a2t, where m1 was the fraction of the substrate cleaved at rate m1, the initial rate of cleavage, m2 = the fraction of the substrate cleaved at rate m2, and m3 was the terminal rate of cleavage.

Mice and macrophages

Six-week-old female C57BL/6NCR mice were maintained as specific pathogen free in autoclaved cages in a laminar flow hood and given sterilized water to minimize “spontaneous” activation of macrophages. To produce responsive macrophages, 2 ml of sterile fluid thioglycollate broth (Difco, Detroit, MI) was injected i.p. After three days the resulting peritoneal exudate cells (PEC) were obtained by lavage with HBSS and plated at 5 × 104 cells/well in 96-well plates with Eagles minimal essential medium (EMEM) and 10% heat inactivated FBS. After 90 min, the wells were washed to remove nonadherent cells. The resulting cultures were 97% macrophages as determined by morphology and staining for nonspecific esterase.

Transfection in vitro

The cytotoxicity of 2,3-dioleoyloxy-N-[2[(peroxycarboxamido)ethyl]-n,n-di-methyl-1-propanaminium trifluoroacetate (DOTPA, Lipofectin, Life Technologies/BRL, Bethesda, MD), N-[1-(2,3-dioleoyloxy)propyl]-n,n,trimethylammonium chloride (DOTMA, Lipofectin, Life Technologies/BRL), or N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP) were mixed at 3:1 (w/w) with dioleoyl phosphatidylethanolamine (DOPE) in chloroform. Mixtures were rotary evaporated to form thin films, placed under vacuum overnight to remove residual chloroform, and hydrated with DMEM by vortex mixing to form a 2 nM lipid emulsion. An equal volume of ribozyme RNA was then added to make a final concentration of 80 µg/ml and the mixture was vortexed. The resulting liposome/RNA complex was removed by centrifugation at 25°C for 30 min before further manipulation. Complexes were then added to macrophage cultures in serum-free DMEM and incubated at 37°C for 3 h. The cultures were gently rinsed once with fresh DMEM and medium containing 10% FBS added. Medium and serum endotoxin levels were <0.1 ng/ml as determined by the supplier; all other reagents contained <0.1 µg/ml endotoxin as quantified by the Limulus amebocyte lysate assay.

6 Abbreviations used in this paper: PEC, peritoneal exudate cell; DMRIE, 1,2-dimyristoyl-rac-glycerol-3-phosphatidylcholine; DOPE, dioleoyl phosphatidylethanolamine; DOTPA, N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride; DOTMA, N-[1-(2,3-dioleoyloxy)propyl]-n,n,trimethylammonium chloride; EEMD, Eagle’s minimal essential medium.
Transfection in vivo

Transfection reagents were prepared as described above and also included 1,2-dimyrystilxylopyrrol-3-dimethyl-hydroxyethyl ammonium bromide (DMRIE, Vical, La Jolla, CA); DOPE, 1:1 (w/w) and dioctadecylmigidoligocyspermine (DOGS, Transfektam; Promega)/DOPE, 2:1 (w/w). An equal volume of ribozyme was added to achieve 80 pg/µl. After vortexing, 1 ml of normalizing liposomes: RNA complexes were injected i.p. into mice previously treated with thioglycollate. PEC were harvested 3 h later by peritoneal lavage with HBSS. The exudates were plated at 1 × 10^6 cells/well in 96-well plates and allowed to adhere for 2 h before washing with HBSS to remove nonadherent cells.

Quantitation of ribozymes in cells and organs

Ribozymes, labeled internally with fluorescein or 32P and injected i.p., as described above, were quantitated in peritoneal macrophages and organs radiometrically. After various intervals, mice were asphyxiated with CO2, peritoneal cells were harvested by lavage, and ribozymes were counted as described above. Spleen, kidney, liver, intestine, lung, and pancreas were also harvested and weighed. Organs were homogenized on ice in a 10-fold excess of RNAZol (Tel-Test), as described by the manufacturer, to obtain total RNA. Fractions were assessed by scintillation counting in each step of the isolation to determine the efficiency of RNA recovery. The RNA isolation process resulted in recovery of >95% of the 32P from solid tissues and >95% from peritoneal exudate macrophages. The extracted RNA was separated by electrophoresis through 10% sequencing-size polyacrylamide gels containing 7 M urea as described above. The gels were run approximately half way such that no material was lost into the lower buffer tank. An aliquot of 32P-labeled ribozyme, which had not been injected, was used as a standard. Gels were exposed to storage phosphor screens overnight. The screens were quantitated using Imagequant software (Molecular Dynamics). Bands comigrating with the ribozyme standards and the total activity in each lane were quantitated. The fraction of ribozyme intact for each sample was determined by dividing the integrated density from the intact band by the integrated density of the entire lane. The total amount of intact ribozyme in each tissue was derived from the measured radioactivity by the following formula: (10 × DPM × (Mol/DPM)) × fraction intact weight of tissue.

Cytokine production and quantitation

After transfection, macrophages isolated as described above were treated with 1 μg/ml polyinosinic:polyribocytidylic acid (Poly I: C) (Pharmacia). After 4 h, 1% formaldehyde (16). The gel was then stained with ethidium bromide, and transferred to Nytran membranes (Schleicher & Schuell, Keene, NH). The resulting blots were probed with 32P-labeled murine TNF-α CDNA probe prepared by random priming according to the manufacturer’s protocol (Pharmacia). Autoradiography was performed without enhancer screens to maximize resolution of individual bands.

Assessment of reagent toxicity

Following ribozyme/lipid treatment of macrophages and harvesting of supernatants, viability of the cells was assessed by incubation with 5 mg/ml of MTT. This compound is reduced by the mitochondrial dihydrogenases, the activity of which correlates well with cell viability (22). After 12 h, the absorbance of reduced MTT was measured at 585 nm.

Statistical analysis

Differences between treatments were tested for significance using Student’s t test. A p value of 0.05 or less was considered significant.

Results

Kinetic parameters of ribozymes tested

Several permutations of the hammerhead ribozyme motif were evaluated for potential use with endogenous expression systems. Several alterations were tested because we have previously determined that excess RNA beyond the binding arms of the hammerhead ribozyme was detrimental to catalytic activity in vitro (16). Thus, both the self-processing and minimonomer versions were designed to splice themselves from longer transcripts, as might be required for expression from gene therapy vectors in vivo. The self-processing pathways are illustrated in Fig. 1. The ability of ribozymes to cleave the target RNA sequence was evaluated and the initial rates are summarized in Table I. The kinetic data were fit to double exponential decay curves; the rates observed for the first, more rapid phase of the cleavage reaction are reported. Rz254 initially cleaved 39% of the substrate with a rate of 0.17/min. The addition of the self-processing domain to rz254sp increased the initial fraction cleaved to 66% with a rate of 1.42/min. Rz442 initially cleaved 36% of its substrate with a rate of 0.48/min. Addition of a self-processing domain to rz442sp reduced the initial fraction cleaved and the rate slightly. Rz442, which was inserted into the variable domain of an autocleaving hairpin ribozyme, rzmm442/24, also had a reduction in the initial fraction cleaved and the initial rate of cleavage to 23% and 0.16/min. The initial rate of cleavage for the ribozyme with the number of target recognition nucleotides reduced to 10, rzmm442/10, was at least 100-fold less catalytic than that of rzmm442/24. Controls, the mutant versions of the above ribozymes, had no detectable cleavage activity (data not shown).

Ribozyme delivery to peritoneal macrophages in cell culture

Initially, we sought to determine whether cationic lipid could be used to deliver intact ribozymes to primary macrophages. For that, ribozyme RNA was prepared by in vitro transcription from the appropriate DNA templates. Murine peritoneal macrophages were treated in vitro with cationic lipid/RNA complexes at a charge ratio of 1:1. After 3 h, the complexes were removed and macrophages washed with HBSS containing RNase A to remove ribozymes on the outside of the plasma membrane. Fig. 2 shows autoradiograms of ribosome RNA extracts from murine peritoneal macrophages after various periods of time. Based on scintillation counting of the ribozyme bands cut from the gel, ~10^4 ribosome molecules per cell were recovered 3 h after transfection. This represented ~5% of the ribozyme added in vitro. More than 50% of the ribozymes remained intact 24 h after the end of the 3-h transfection period. Therefore, the intracellular half-life could not be precisely determined, but was longer than 24 h.

Specific inhibition of TNF-α secretion after ribozyme treatment

After in vitro transfection, macrophages were stimulated with LPS to induce TNF-α production. Fig. 3 illustrates inhibition of TNF-α production after treatment with DOTMA:DOPE complexed with various ribozymes directed to site 254 or 442 of murine TNF-α.
mRNA. Eight hours after LPS stimulation, the self-processing ribozymes, rz254sp and rz442sp, inhibited macrophage TNF-α secretion by 80%. In contrast, an irrelevant ribozyme targeted to human stromelysin inhibited secretion of TNF-α by 34%, a level similar to another control with lipid only. Rz254sp and rz442sp were the only constructs relative to their inactive homologues to significantly inhibit TNF-α secretion. Removal of the self-processing domains reduced the ability of rz254 and rz442 to inhibit TNF-α. After removal of those domains, catalytic activity did not differ from their inactive controls. Incorporation of rz442 into a minimonomer, rzmm442/24, did not change its inhibitory activity. Active and inactive versions of rzmm442/10, containing 10-bp recognition domains, inhibited TNF-α secretion by 55% relative to sham-treated cells. However, when compared with cells treated with irrelevant ribozyme or lipid only, there were no significant differences.

To better characterize the inhibitory activities of some of the ribozymes tested, a dose response experiment was performed with the active rz254sp, the inactive rz254spd, or an irrelevant ribozyme (Fig. 4A). Rz254sp at 17 nM (IC₅₀ = 10.7 nM) inhibited 50% of the TNF-α secretion, a 58% lower concentration than required for the inactive ribozyme (IC₅₀ = 24 nM). The inactive ribozyme inhibited significantly (p = 0.05) more TNF-α secretion than the irrelevant ribozyme at 51 nM. This finding indicates a possible base pairing sequence-specific effect not due to the catalytic activity of the ribozyme. The irrelevant ribozyme also inhibited some TNF-α secretion (IC₅₀ = 56.3 nM). To assess target specificity, macrophage IL-1α production after LPS-stimulation was also measured. IL-1α secretion was not altered by the concentrations of cationic lipid/ribozyme treatments used in these experiments (Fig. 4B).

Table I. Initial rate and fraction of substrate cleaved by ribozyme

<table>
<thead>
<tr>
<th>Ribozyme</th>
<th>Initial Rate</th>
<th>Substrate Amount Cleaved at the Initial Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rz254</td>
<td>0.17 ± 0.051/min</td>
<td>0.39</td>
</tr>
<tr>
<td>Rz254sp</td>
<td>1.42 ± 0.134/min</td>
<td>0.66</td>
</tr>
<tr>
<td>Rz442</td>
<td>0.48 ± 0.042/min</td>
<td>0.36</td>
</tr>
<tr>
<td>Rz442sp</td>
<td>0.27 ± 0.073/min</td>
<td>0.25</td>
</tr>
<tr>
<td>Rzmm442/10</td>
<td>0.001 ± 0.001/min</td>
<td>ND</td>
</tr>
<tr>
<td>Rzmm442/24</td>
<td>0.16 ± 0.003/min</td>
<td>0.23</td>
</tr>
</tbody>
</table>

* Single turnover conditions were assessed with 40 nM ribozyme and 1 nM substrate. Rate = mean ± SD (n = 3); data are shown for one representative experiment of three.

FIGURE 1. Schematics of self-processing ribozymes. A. Rz254sp was designed to process itself from a longer transcript, such as could be made efficiently by RNA polymerase III. The ribozyme was designed to be placed near the 3' end of the transcript and cleave the 5' portion at the site of the arrow. B. Rzmm442/24 was designed to cleave excess RNA both 5' and 3' of the ribozyme domain. C. The ribozyme then circularized so as to protect the ends from exonuclease activity.
Because toxicity of the cationic lipids or the lipid/RNA complex could lead to a reduction of TNF-α production, lipid alone, lipid/RNA, or lipid/RNA and LPS were assessed for alteration in cell viability. DOSPA:DOPE alone or in combination with any of the ribozymes tested at concentrations to 114 nM were not toxic as measured by the MTT assay for mitochondrial enzyme activity. Concentrations >114 nM inhibited macrophage reduction of MTT, indicating some toxicity (data not shown). LPS either alone or in combination with DOSPA, DOTMA, DOGS:DOPE, DMRIE:DOPE or ribozyme were more cytotoxic than the cationic lipid/ribozyme complex without LPS.

Intraperitoneal accumulation of ribozymes in macrophages and other sites

A significant challenge for use of preformed ribozymes in vivo is to achieve sufficient ribozyme concentrations within cells following systemic administration. Fig. 5 demonstrates macrophage accumulation of intact ribozymes following i.p. administration of a cationic lipid/ribozyme complex. Peritoneal macrophages accumulated ~3 × 10⁶ intact ribozyme molecules per cell at the concentration of DOSPA:DOPE/ribozyme tested, or ~6% of the administered ribozyme. They were distributed within the nuclei and cytoplasm of the majority of adherent macrophages 2 h after administration of the DOSPA:DOPE/ribozyme complex as detected by fluorescence microscopy (Fig. 6). With the cytofectins, DMRIE:DOPE, ~3 × 10⁵ intact ribozymes accumulated per cell, and with DOTMA ~1.5 × 10⁴ intact ribozymes per cell. Ribozymes administered without cationic lipid were not detectable within the macrophages. Spleen, kidney, liver, intestine, lung, and pancreas also accumulated some intact ribozyme when delivered as a complex with DMRIE:DOPE or DOSPA:DOPE. Those organs accumulated between 0.25 and 1.0% of the administered dose.

Inhibition of TNF-α secretion following i.p. ribozyme administration

To assess ribozyme efficacy following in vivo transfection, peritoneal macrophages were harvested 3 h after i.p. ribozyme administration and TNF-α secretion assessed by specific ELISA (Fig. 7A). Macrophages from mice treated with rz254sp produced 70%
less TNF-α than macrophages from control mice treated with only HBSS. The inactive ribozyme, rz254spd, reduced TNF-α expression by 37%. Neither DOSPA:DOPE alone nor complexed with an irrelevant ribozyme were significantly (p > 0.05) inhibitory. There were no significant (p > 0.05) differences in the levels of IL-1α secreted by the same cell among the various treatment groups (Fig. 7B).

Accumulation of TNF-α degradation products after ribozyme treatment

If suppression of TNF-α secretion was due to cleavage of the TNF-α mRNA by the ribozymes, then mRNA cleavage products should be present within the cells. Therefore, Northern blot analysis was conducted with total cellular RNA extracted from macrophages 8 h after LPS stimulation or 11 h posttransfection (Fig. 8). Mature TNF-α mRNA should be ~1800 bases. Rz254sp should cleave the mRNA into fragments of ~250 and 1550 bases, and rz442sp into fragments of ~440 and 1360 bases (16). Macrophages from mice treated with the active ribozymes, rz254sp or rz442sp, had TNF-α mRNA degradation products of ~100 or 200 bases. RNA from all macrophages, which were treated with either active or inactive ribozyme, but not irrelevant ribozyme or control, had an additional hybridization product at ~2.6 kb for active ribozyme, or 3.0 kb for inactive ribozyme. Those are within the correct size range for primary transcripts of the TNF-α gene, 2960–3000 bases including polyadenylation.

PEC recruitment after treatment with cationic lipid/ribozyme complexes

The number of adherent peritoneal cells recovered from mice increased after treatment with 40 μg of rz442 complexed with...
TB

DOSPA:DOPE, DMRIE:DOPE, or DOTMA:DOPE at a 3:1 charge ratio. Each of the complexes induced approximately a 400% increase in the number of peritoneal exudate cells recovered 24 h after treatment (Fig. 9).

**Discussion**

We have demonstrated that anti-TNF-α ribozymes can inhibit TNF-α secretion in a sequence-specific and concentration-dependent manner. Furthermore, cationic lipids can be used to deliver these potentially therapeutic RNA molecules to cells in vitro and in vivo. After controlling for nonspecific inhibition due to toxicity of the cationic lipid/RNA complex, we have shown that ribozymes targeted to different sites of the TNF-α mRNA inhibited TNF-α production in culture. We now demonstrate the transfection of ribozymes into macrophages in vivo resulted in suppression of TNF-α production. Ribozymes accumulated in macrophages following i.p. administration were biologically active and inhibited TNF-α secretion by ~70%. Rz254sp and rz442sp both showed better inhibitory activity than their inactive controls, demonstrating that catalytic activity was required for maximal suppression. Irrelevant ribozyme did not significantly inhibit the cytokine secretion. Ribozymes delivered with DOSPA:DOPE to murine peritoneal macrophages as complexes were effective in suppressing TNF-α gene expression for a 24-h period.

The cleavage rates of the ribozymes tested in this study varied greatly under single turnover conditions. For rz442, addition of more sequence beyond the substrate recognition domains reduced the initial rate of catalysis. That may be due to improper folding of a larger structure, which could result in reduced, or no, cleavage of the substrate. In contrast to rz442sp, the catalytic activity of rz254sp was unexpected because with single turnover conditions the catalytic rate of rz254sp was faster than rz254. The latter had no additional sequences beyond the binding arms. The 5’ half of the self-cleavage domain should compete with substrate for the binding arms, slowing the cleavage rate of rz254sp relative to rz254, but that did not occur in our experiments. This kinetics issue is being explored in additional studies.

Embedding rz442 within the minimonomer construct served two theoretical purposes. First, the covalently closed circle formed by the minimonomer should be stable to exonucleases, thus enhancing the in vivo half-life of the molecule. However, we were unable to demonstrate a greater half-life of rzmnm442/24 within the time period studied, as rz442sp was quite stable in primary macrophages. Second, the minimonomer domain could act as a small, highly structured RNA carrier, which would allow self-processing from a longer transcript. However, the catalytic activity of rzmnm442/24 was reduced by 66% relative to rz442. It is possible that further refinement of the 5’ and 3’ junctions of the hammerhead ribozyme within the minimonomer will allow for greater activity.

Our study of intracellular stability indicated that the ribozymes were quite stable when delivered as cationic-lipid complexes to cultured macrophages. Moreover, there was little additional self-processing activity observed for rz442sp after forming complexes with cytofectins. This may indicate that most of the ribozyme remained sequestered as a complex with the cationic lipid within the endosomes. This possibility is supported by the light micrographs which show a nonuniform cytoplasmic distribution of the ribozyme consistent with its association with the endosomes.

Ribozymes targeted to sites 254 and 442 of murine TNF-α mRNA were able to inhibit TNF-α secretion significantly better than their inactive counterparts or an active unrelated ribozyme. This finding demonstrates the requirement of catalytic activity for maximal inhibition. However, the inactive and irrelevant controls had some inhibitory activity. Up to 39% of the total inhibition observed for rz254sp and rz442sp was probably due to toxicity and other nonspecific causes. Enhanced inhibition by the inactive compared with the irrelevant ribozyme indicated that the inhibition of TNF-α secretion by rz254sp was partially due to a sequence-specific activity of the inactive ribozyme independent of its catalytic activity.

A dose-response study was used to evaluate the relative potencies of rz254sp, rz442sp, as compared with the irrelevant ribozyme. The lipid/ribozyme complexes used did not significantly inhibit IL-1α secretion, indicating that catalytic activity was potentially specific for TNF-α. The relative activities of the oligonucleotides indicated that the nonspecific, sequence specific, and ribozyme specific activities could be distinguished, but does not account for the sequence-specific, non-ribozyme effect. Inhibition due to an antisense effect was improbable because the inactive ribozyme
The selective destabilization of mRNA containing concatenated AUUUU motifs, of which TNF-α is an example (31), require CAP binding and translation for destabilization (32). In our study, all of the ribozymes cleaved such that the CAP and 5′ untranslated region of the TNF-α mRNA were separated from the protein coding sequence and 3′ untranslated region, including the AUUUU destabilizing element. The removal of all sequences required for interaction with ribosomes should exclude the remainder of the mRNA from selective degradation mediated by the AUUUU element. Exonuclease activity 5′ to 3′ would then be required to degrade the 3′ cleavage product. Although uncapped mRNA trans- fected into cells may not be translated efficiently, they are not necessarily degraded rapidly (33).

We have demonstrated that hammerhead ribozymes complexed with cytofectins can suppress TNF-α production. Ribozymes delivered with DOSPA:DOPE and DOGS:DOPE were stable in activated macrophages. Recruitment of inflammatory cells into the peritoneal cavity by three different formulations of cationic lipids indicates that these delivery vehicles may create a proinflammatory environment when combined with ribozymes. Further studies will be required to modify the delivery formulations such that the size, protein binding characteristics, and lipid composition do not promote recruitment of inflammatory cells.

Acknowledgments
We thank Drs. George Breuning and Neil E. Hubbard for helpful discus sions and advice.

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was too short to interrupt the progress of ribosome translocation along the mRNA (23), and chemically incapable of stimulating RNase H to degrade the mRNA as would antisense DNA (24).

Rz254, rznm442/10, and rznm442/24 were unable to inhibit TNF-α secretion in a catalytically dependent manner. The inhibition observed for these ribozymes was either nonspecific or a combination of nonspecific and sequence-specific non-ribozyme effects. Rz254 was very similar in sequence to another ribozyme, mRz1, reported to inhibit TNF-α by 49.1% in culture at a concentration of 5 µM when complexed with the cytotoxicin, DOTAP (25). We also found this same ribozyme to inhibit TNF-α by 48.9%, but the inactive control also inhibited activity by 48.6%. Neither of these ribozymes were significantly more inhibitory than DOTMA:DOPE alone or the irrelevant ribozyme complexed with DOTMA:DOPE. Ribozymes mutated in the catalytic domain were not used as controls in the previous study (25). That control combined with the lack of statistical analysis may indicate that inhibition of TNF-α secretion by a ribozyme-dependent mechanism was not previously demonstrated. The catalytic activity of rz254 was improved by the addition of the self-processing domain in rz254sp. This may be due to relief of alternative secondary structures which can be predicted to form between the 5′ binding arm and the ribozyme core in rz254.

Different cationic lipid formulations varied greatly in their ability to facilitate ribozyme uptake by peritoneal macrophages in vivo. DOTMA:DOPE was useful as a delivery reagent for primary macrophages in culture. In vivo, however, DOSPA:DOPE enhanced uptake of intact ribozyme by peritoneal macrophages by 10-fold. That increase may be due to the multiple positive charges associated with the spermine head group of DOSPA vs the single positive charge associated with the head group of DOTMA.

Treatment of macrophages with anti-TNF-α ribozymes in vitro and in vivo resulted in TNF-α mRNA degradation products. In addition, transfection with both active and inactive ribozyme, but not irrelevant ribozyme, resulted in the accumulation of primary transcripts of the TNF-α gene. That observation differs from what others have reported for plants (26) or mammals (13–15). The cleavage products were probably not produced during isolation of the cellular RNA, as the macrophages were lysed in the presence of guanidine isothiocyanate and EDTA. In addition, excess EDTA was present throughout RNA isolation and electrophoresis. Our previous studies have shown that cleavage of TNF-α mRNA by rz254sp or rz442sp among total cellular RNA in vitro requires at least 10 mM Mg2+ and extended incubation at 37°C (16). The observed 3.0 Kb TNF-α transcript may be due to the 2766-base transcript may be due to the 2766-base untranslated region, including the AUUUA de-


