Construction of a Ribozyme Directed Against Human Interleukin-6 mRNA: Evaluation of Its Catalytic Activity In Vitro and In Vivo

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We have designed a ribozyme (Rz) that cleaves human interleukin-6 (IL-6) mRNA in vivo. This Rz was tested in vitro, and was found to give expected size fragments. It was then incorporated into a mammalian expression vector containing the constitutive cytomegalovirus (CMV) immediate early promoter and transfected into human U amniotic cells. Cell clones that stably express this catalytic RNA have been obtained. Some of them displayed a marked reduction of tumor necrosis factor (TNF)-induced IL-6 production. Their reduced ability to express IL-6 was related to the amount of Rz they produced and to the extent of IL-6 mRNA cleavage as observed by a ribonuclease protection assay. These data provide a method to study further the role of IL-6 production in various biologic situations, and suggest the feasibility of developing Rzs directed against various cytokines to study their biologic role and mechanism of action.

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INTERLEUKIN-6 (IL-6) is a pleiotropic cytokine mainly involved in the regulation of inflammatory and immunologic processes. Among its major roles, it triggers the production of acute-phase proteins by hepatocytes, activates proliferation and differentiation of cytotoxic T cells, and stimulates the terminal differentiation of B lymphocytes; it is also a growth factor for murine hybridoma and plasmacytoma cell lines. Furthermore, it has been implicated in several pathologies as a potent growth factor (perhaps among others), for example, in multiple myeloma and in Kaposi’s sarcoma. In this study, we have attempted to develop a ribozyme (Rz) capable of specifically cleaving human IL-6 mRNA in vitro and in vivo, and therefore inhibiting IL-6 production.

Rzs are catalytic RNAs that have the ability to cleave the phosphodiester bond of target RNA(s). The simple hammerhead Rz found in plant viroids and virusoids can be adapted to cleave specifically an RNA sequence of choice. The catalytic domain of the Rz consists of 12 ribonucleotides. The specificity of the Rz is determined by short flanking sequences complementary to the target sequence.

Several reports indicate that the hammerhead type of Rz functions in living cells, Cotten and Birnstiel and Cameron and Jennings reported Rz-mediated inhibition and lowering of specific gene expression in Xenopus laevis oocytes and monkey (COS-1) cells, respectively. Sarver et al. showed that an Rz directed against human immunodeficiency virus type 1 (HIV-1) gag RNA reduced p24 antigen expression in CD4+ HeLa cells. A hammerhead Rz is also active in bacterial cells. In this study, an Rz directed against human IL-6 mRNA has been designed, characterized, and expressed in UAC amniotic cells.

MATERIALS AND METHODS

Oligonucleotide Synthesis

Synthetic oligodeoxyribonucleotides were prepared on an Applied Biosystems DNA Synthesizer 381A (Maarssen, The Netherlands). The sequences for the oligodeoxyribonucleotides were as follows. (1) Oligodeoxyribonucleotides used to clone the IL-6 Rz DNA into pBluescript (pBS) II SK (Stratagene, La Jolla, CA)—IL-6 Rz 56: 5’-AGCTATGAGTTACACTCGCTAGTCGAGTTCTGAAGGACGAAAC’3’, (flanking Kpn I and HindIII overhangs are underlined); IL-6 Rz 48: 5’-GAACGTGATGACCTGTCGATGCTCGTGGAAGAACTTTGTAAGT-3’. (2) Polymerase chain reaction (PCR) assay primers used to detect the IL-6 Rz in cellular DNA or RNA—Rz 3’: 5’-TGCCACACTAGAGC-ACAGTG-3’; Rz 5’: 5’-GAAGCTACGAACTGACGATCCAGC’3’. (3) PCR assay primers used to detect IL-6 mRNA in cellular RNA—IL-6 3’: 5’-AACTGCTAGACCCATTTCC-3’; IL-6 5’: 5’-GCA-TCTAATGAGGACAGTTG-3’. (4) PCR assay primers used to detect β actin RNA in cellular RNA—actin 3’: 5’-CTCCTTAATGCTACGCAAGATTTC-3’; actin 5’: 5’-GTGGGCGCGACCAG-GAACA-3’.

Plasmid Constructions

The Rz double-stranded DNA obtained by the annealing of the two synthetic single-stranded oligonucleotides IL-6-Rz 56 and IL-6-Rz 48 was cloned into a Kpn I- and HindIII-digested pBluescript II SK pBS IL-6-Rz. The sequence and orientation of the inserts were confirmed by dideoxynucleotide sequencing of the construct. For in vivo assays, the IL-6-Rz was cloned into the pRC/CMV mammalian expression vector (Invitrogen, Oxon, UK) downstream to the constitutive CMV immediate early promoter and was followed by the SV40 polyadenylation signal (pRC/CMV-IL-6-Rz).

In Vitro Transcription of Rz and IL-6 mRNA

The transcription reactions were performed using 1 µg of linearized DNA template in 0.4 µmol/L each of adenosine triphosphate (ATP), guanosine triphosphate (GTP), and cytidine triphosphate (CTP), +0.2 µmol/L cold uridine triphosphate (UTP), 8 µmol/L MgCl2, 2 µmol/L spermidine, 5 µmol/L dithiothreitol (DTT), 40 µmol/L Tris-HCl (pH 8), 25 µmol/L NaCl, and 2 µ/µL T7 RNA polymerase. 1P-labeled transcripts were needed, 15 µCi of [32P]-UTP (3,000 Ci/mmol) was included. The reactions were performed at 37°C for 1 hour, treated for 15 minutes with RNase-free DNase to degrade the template DNA, and the products isolated by phenol chloroform extraction and ethanol precipitation. The amount of labeled transcripts was determined by cold 10% trichloroacetic acid (TCA) precipitation. IL-6 mRNA was transcribed from Sal I-linear-

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**Fig 1.** Design of the Rz directed against human IL-6 mRNA. The IL-6 mRNA target region contains a GUC cleavage site at 510 nucleotides from the cap site on IL-6 mRNA, corresponding to aa 121 of the mature IL-6. The in vitro-transcribed IL-6 mRNA was used as substrate. (---) A vector-derived sequence, which is not present in natural IL-6 mRNA. P1 and P2 are cleaved fragments of this synthetic messenger RNA. The in vitro-transcribed hammerhead IL-6 Rz contains vector-derived flanking sequences, indicated in oblique. Shaded nucleotides represent the catalytic domain.

**Fig 2.** In vitro cleavage of IL-6 mRNA: effect of MgCl2. In vitro cleavage reactions were performed in 50 mmol/L Tris-HCl (pH 8), 1 mmol/L EDTA, and 20 mmol/L MgCl2. The resulting products were analyzed by autoradiography and quantified by densitometric scanning at 500 nm in a DU-8 spectrophotometer (Beckman, Palo Alto, CA). The extent of cleavage products was determined as P1 + P2/P1 + P2 + S.

**Rz-Mediated mRNA Cleavage In Vitro**

Reactions were performed in 50 mmol/L Tris-HCl, pH 8, and 20 mmol/L MgCl2 (sometimes in the presence of human placental ribonuclease inhibitor). Reactants (Rz and target RNA) were mixed together at the desired molar ratios and the reactions were initiated by adding MgCl2. The reactions were stopped by the addition of an equal volume of 95% formamide, 20 mmol/L EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol FF. Reaction products were then separated on a 4% polyacrylamide/8-mol/L urea gel. The substrate and cleavage products were visualized by autoradiography and quantified by densitometric scanning at 500 nm in a DU-8 spectrophotometer (Beckman, Palo Alto, CA). The extent of cleavage products was determined as P1 + P2/P1 + P2 + S.

**Stable Transfection of IL-6 Rz in UAC Cells**

UAC human amniotic cells were kindly provided by Dr K. Cantell (Helsinki, Finland). These cells produce IL-6 after tumor necrosis factor-α (TNF-α) induction as described by Snyers and Content. Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal calf serum, penicillin, and streptomycin at 37°C and 5% CO2. Ten micromolars of unlabeled pRc/CMV IL-6 Rz plasmid was transfected into cells by calcium phosphate precipitation. Before transfection, the construct was purified on a Qiagen anion-exchange column (Westburg, Leusden, The Netherlands). Multiple stable neomycin-resistant clones were isolated.

**IL-6 Bioassay**

IL-6 activity was measured using the IL-6-dependent mouse hybridoma cell line 7TD1. Briefly, 7TD1 cells were cultivated in flat-bottom microwell plates that contained 2 × 104 cells per well in the presence of serial dilutions of supernatant. After 4 days of culture, the number of surviving cells was determined by a colorimetric assay for hexosaminidase. In this assay, the absorbancy is proportional to the number of cells present in each culture. IL-6 activity was expressed in laboratory units (LU) per milliliter; 1 LU is defined as the dilution giving half the maximal proliferation level of 7TD1 cells. One unit corresponds to approximately 1 pg of IL-6. Interassay variability was corrected by the use of a recombinant IL-6 internal standard. Each sample was tested in triplicate, and the corresponding means and standard deviations are presented.

**PCR**

RNA and DNA extraction. Total cellular RNA was obtained using the rapid guanidinium thiocyanate method as described by Chomczynski and Sacchi. An RNAzol solution was supplied by...
Cinna-Biotex Laboratories (Houston, TX). For genomic DNA extraction, cells were washed two times in phosphate-buffered saline (PBS) and treated with 0.01 mol/L Tris (pH 7.4), 0.15 mol/L NaCl, 1 mmol/L EDTA, 0.5% sodium dodecyl sulfate (SDS), and 0.5 mg/mL proteinase K at 37°C for 30 minutes. DNA was then isolated (PBS) and treated with 0.01 mol/L Tris (pH 7.4), 0.15 mol/L NaCl, 1 mmol/L EDTA, 0.5% sodium dodecyl sulfate (SDS), and 0.5 mg/mL proteinase K at 37°C for 30 minutes. DNA was then isolated (PBS) and treated with 0.01 mol/L Tris (pH 7.4), 0.15 mol/L NaCl, and 0.1% SDS, and precipitated after phenol-chloroform extraction.

Reverse transcription. Reactions were performed by mixing 4 μg of total RNA from the various transformants with 0.01 mol/L Tris (pH 7.6), 0.15 mol/L NaCl, 1 mmol/L EDTA, 0.5% sodium dodecyl sulfate (SDS), and 0.5 mg/mL proteinase K at 37°C for 30 minutes. DNA was then isolated by phenol-chloroform extraction and ethanol precipitation.

PCR reactions. One quarter of the cDNA obtained from the RT reaction (or 5 to 50 ng of genomic DNA) was incubated in presence of 2 U of Taq polymerase (Promega, Leiden, The Netherlands) in 0.25 mmol/L of each dNTP(s), 10 pmol of each 5' end primers, 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 10 mmol/L Tris-HCl (pH 9 at 25°C), and 0.1% Triton-X-100. Reaction mixtures were overlaid with mineral oil and placed in a DNA thermal cycler for 20 to 40 cycles (94°C for 1 minute, 60°C for 1.5 minutes, and 72°C for 2 minutes). The PCR-amplified products were analyzed by electrophoresis in a 1% agarose gel in Tris-acetate EDTA buffer and blotted to a nylon membrane. Blots were hybridized to a specific 32P-labeled oligonucleotide probe (IL-6.Rz 48) at 68°C in 6× SSC (1× SSC = 0.15 mol/L NaCl + 0.015 mol/L sodium citrate), 5× Denhardt’s solution, 1% SDS, and 100 μg/mL herring-sperm DNA. Membranes were washed three times in 2× SSC and 0.05% SDS for 15 minutes at room temperature, and once in 2× SSC and 0.1% SDS for 30 minutes at 65°C.

**RESULTS**

Rz-Mediated Cleavage of IL-6 mRNA In Vitro

A hammerhead Rz directed at the GUC sequence located at 510 nucleotides of IL-6 mRNA (Fig 1) was introduced into a Bluescript II SK plasmid. With this construct as template, IL-6 Rz was synthesized in vitro by T7 RNA polymerase.

In vitro cleavage of IL-6 mRNA by this IL-6 Rz was demonstrated directly using a labeled IL-6 mRNA substrate. The sizes of the fragments produced by the ribozyme cleavage were 662 (P1) and 379 (P2) nucleotides as predicted by the location of the single site of cleavage (Fig 2A, P1 and P2). As expected, cleavage did not occur without MgCl₂ (Fig 2A, lane 1). In this experiment, cleavage reactions were performed for 1 hour in 20 mmol/L MgCl₂ at 50°C. To characterize further these Rz’s activities in vitro, additional assays were conducted. Various Mg²⁺ concentrations were tested. Figure 2B shows that the percentage of cleavage obtained after 30 minutes at 37°C increased with the MgCl₂ concentration from 2 mmol/L to 40 mmol/L; it varied from approximately 23% to 32% for MgCl₂ concentrations of 10 mmol/L and 40 mmol/L, respectively. These high Mg²⁺ optimal concentrations are consistent with hammerhead Rz’s...
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A CMV Poly A signal

\[\text{Rz}^{5'} \quad \text{128 bp} \quad \text{Rz}^{3'}\]

Fig 4. PCR detection of IL-6 Rz in DNA or RNA from permanently transfected UAC clones. (A) Primers for the PCR. One primer (Rz 5') contains a sequence corresponding to the junction between bacteriophage T7 RNA polymerase promoter and the Rz. The other primer (Rz 3') contains a sequence in the bovine growth hormone (BGH)-derived polyA signal-containing sequence. (B) Detection of the IL-6 Rz from cellular DNA by PCR. The expected size of the PCR-amplified product is 128 nucleotides (arrow). Lanes 4 through 7 represent DNAs amplified from genomic DNA obtained from the various transformed clones. Lane 3 corresponds to UAC untransfected cells. C+ (positive control) is pRc/CMV-IL-6.Rz plasmid. C- (negative control) is SP65T26K-plasmid. Amplified products were separated by electrophoresis in an agarose gel and blotted to a nylon membrane. Hybridization with a 5' end-radiolabeled Rz-specific oligonucleotide (IL-6 Rz 481 was performed as described in Materials and Methods. For semiquantitative conditions of the PCR reaction, 30 ng of genomic DNA and 40 cycles (94°C for 1 minute, 60°C for 1.5 minutes, 72°C for 2 minutes) have been used. (C) Detection of IL-6 Rz from total cellular RNA by RT-PCR. Conditions are identical to those described in Fig 4B. Total RNA extracted from the various clones is submitted to an RT reaction with the antisense primer before the actual PCR (see Materials and Methods). No amplification was observed with extracted RNA without RT reaction (not shown).

activities observed in previous reports. Moreover, it was observed that this Rz was active at 15, 20, and 60°C. At 15°C, substrate cleavage was less efficient and no cleavage was detected at 4°C. At 60°C, RNA was also unspecifically degraded (data not shown). Several experiments were performed varying the IL-6 substrate to Rz ratio (data not shown). No cleavage was observed with excess of substrate. For an Rz to IL-6 mRNA ratio of 10:1, cleavage was slightly detectable after 1 hour at 37°C. Cleavage increased with increasing Rz to mRNA IL-6 ratios and leveled off beyond a ratio of approximately 50 to 100:1. The plot of percentage of product versus time (Fig 3) also demonstrates that for an Rz to IL-6 mRNA ratio of 14:1, more than one third of the IL-6 mRNA was approximately cleaved within 15 minutes. Almost 80% of the IL-6 mRNA was cleaved after 2 hours of incubation at 37°C.

IL-6 Rz Activity In Vivo

DNA encoding the IL-6 Rz was cloned into a pRc/CMV plasmid under the control of the constitutive CMV immediate early promoter (pRc/CMV.IL-6 Rz). As it has vector-derived sequences at its 5' end and a polyA tail at its 3' end, this in vivo-expressed Rz is structurally different from the in vitro Rz. Amniotic UAC cells were transfected with this construct by the calcium phosphate precipitation method. A large number of neomycin-resistant clones were obtained and further analyzed, but only four of them are described here (R3, R9, R16, and R21). To demonstrate that the Rz DNA was indeed present and expressed in transfected cell clones, total DNA and RNA from different clones were submitted to a PCR amplification assay using specific primers (Fig 4A). IL-6 Rz DNA (Fig 4B) was detected in each clone, whereas none was detected in untransfected UAC cells (lane 3). As shown in Fig 4, clones varied in the amount of amplified DNA. For example, clones R3 and R9 (lanes 4 and 5, respectively) showed less amplification of DNA than clones R16 and R21 (lanes 6 and 7), probably representing differences in copy number. Furthermore, clones that presented less DNA amplification also had less Rz expression (Fig 4C).

To evaluate the effect of IL-6 Rz on its target mRNA in vivo, a PCR amplification using primers specific to IL-6
mRNA was performed (Fig 5A and B). When untransfected UAC cells were not induced for IL-6 production, a low basal level of IL-6 mRNA (indicated by a PCR product of 596 bp) was nevertheless detected (Fig 5B, lane 3). The clone R3 (lane 4), with the low expression of Rz as demonstrated in Fig 4C, presented a level of IL-6 mRNA expression similar to that of untransfected induced UAC cells (lane 2). However, when the Rz was highly expressed, as demonstrated for clones R16 and R21, the level of IL-6 mRNA was only slightly higher than the basal rate (lanes 5 and 6). The actin PCR amplification (lower panel) shows no significant differences in the amount of total RNA amplified.

After evaluation of IL-6 mRNA expression, the clones were tested for IL-6 production following induction with different concentrations of TNF (Fig 6A). Only two TNF doses are presented in Fig 6B. The extent of inhibition observed between the clones was reproducible in various experiments. In summary, IL-6 production, in clones presenting higher Rz expression (R16, R21), was decreased as compared with UAC control, whereas low producing clones (R3, R9) displayed a normal level of IL-6 production. Furthermore, the higher the level of IL-6 production, the lower the relative effect of the Rz (Fig 6A). Indeed, induction of IL-6 by low doses of TNF (0.1 to 0.3 ng/ml) resulted in greater than 97% inhibition of IL-6 activity. With higher TNF doses (20 to 30 ng/ml), the inhibition reached only 85% to 90%.

Seemingly, high Rz to substrate ratios are required for Rz inhibitory function in vivo, as previously suggested.11 Inversely, in Fig 6B, the relative increased effect of the Rz observed with higher TNF inductions is probably due to the lower production of IL-6 obtained in this experiment. For TNF induction of 3 ng/ml, the IL-6 production observed in UAC was approximately 2,000 U/ml in Fig 6A, compared with 386 U/ml in Fig 6B. Additionally, clones expressing high levels of IL-6 Rz and low levels of IL-6 protein at 4 months of culture (R16, R21) presented an IL-6 production similar to that of untransfected cells at 6.5 months. Additional experiments showing that expression of the Rz RNA and presence of the construct (assayed by PCR) declined between 4 and 6.5 months after isolation of the clones suggest that the inhibition in IL-6 expression observed in some clones UAC is, in fact, dependent on the Rz expression (data not shown).

To demonstrate that the Rz effect observed on the IL-6 messenger RNA expression was due at least partly to its catalytic activity (and not only to an antisense effect), in vivo cleavage products were searched after Rnase protection with a 274-nucleotide antisense probe covering the site of interaction and cleavage by the IL-6 Rz. As demonstrated in Fig 7A, the expected, protected 5' (pP2), and 3' (pP1) cleavage products of 75 and 199 nucleotides, respectively, are detected, in the left panel of Fig 7B, in TNF-induced Rz-containing cells (lane 8), and in the cell-free Rz reaction (lane 2), whereas the target RNA in control transfections (no Rz, lanes 4 and 5) remains unaffected. Only clone R16 is represented in this panel and similar protections have demonstrated an IL-6 mRNA cleavage in clone R21, but not in clones R3 and R9, as expected (lanes 7, 4, and 5). In parallel with the decreased expression of Rz in clone R16 after 6.5 months of culture (data not shown), we observed no more in vivo cleavage in these cells under the same conditions (lane 7 of the left panel). Taken together, all the above-described in vivo experiments prove that the decrease of IL-6 production and of its mRNA expression correlate with an IL-6 Rz catalytic activity.

![Figure 5: RT-PCR detection of IL-6 mRNA from total RNA extracted from stable transfectants. (A) Primers used for the IL-6 mRNA amplification. (B) PCR reaction. In the upper panel, the PCR-amplified IL-6 cDNAs were separated by electrophoresis in a 1% agarose gel. The expected size of amplified product is 596 nucleotides (arrow). Molecular weight was evaluated by comparison with fragments of Hae III-digested dX174 DNA (lane 1). Lane 2, UAC I corresponds to untransfected UAC cells treated for 6 hours with 3 ng/ml TNF-α. Lane 3, UAC II corresponds to untransfected cells without IL-6 induction. Lanes 4, 5, and 6, UAC cells have been transfected with the Rz-containing construction pRc/CMV-IL-6 Rz and treated with TNF-α as UAC I. Lane 7, negative control without RT. Lane 8, C+ positive PCR control: 1 ng of pEMBL7/IL-6 DNA. The semiquantitative conditions have been obtained with 4 μg of total RNA and 30 cycles (see Fig 4B). The lower panel represents actin mRNA amplification in semiquantitative conditions using specific primers (see Materials and Methods). Lanes in the upper and lower pictures correspond to the same cell lines. The expected size of amplified product is 548 nucleotides (arrow). As the actin mRNA is expressed higher in cells for semiquantitative conditions, only 15 cycles are needed when 4 μg of total RNA is amplified.](https://www.bloodjournal.org/content/123/12/3762)
DISCUSSION

In this study, we provide evidence that an Rz we have constructed to cleave human IL-6 mRNA is effectively active both in vitro and in vivo. It is important to note that the two Rzs, expressed in vitro or in vivo, are structurally different. Main differences of these basically identical Rzs are in the length and composition of the non-base-pairing flanking sequences attributed to a polyA tail. The in vitro transcript Rz counts 15 vector-derived bases at the 5' end, and 12 vector-derived bases at the 3' end. The base-pairing sequences consist in 11 and 13 nucleotides at the 5' and 3' ends of the Rz, respectively. The full length of this transcript is 73 nucleotides (Fig 1). The in vivo Rz has 31 vector-derived bases at its 5' end, and approximately 170 nucleotides and the polyA tail at its 3' end. This corresponds to approximately 250 nucleotides, in addition to the polyA tail. As these extra sequences are part of the Rz transcript, they might affect its catalytic activity, stability, and intracellular localization in vivo. It is then encouraging to find that such a molecule is active within a complex intracellular environment, since in vivo results obtained in this work have shown that the present hammerhead Rz expressed in amniotic cells reduced IL-6 mRNA expression by approximately 80% to 97%. In vitro, the maximum cleavage obtained at 37°C was approximately 80% in 1 hour, with an Rz to substrate ratio of 14:1. This is in contrast to 90% cleavage reported for an oligonucleotide substrate (after 40 minutes at 25°C with a Rz to substrate ratio of 1:25). As our Rz core sequence is the same as in the usual hammerhead Rz, the limitation on rate is probably due to the use of a large RNA as substrate. This is consistent with the results of Heidenreich and Eckstein and Ellis and Rogers. In a direct comparison, Heidenreich and Eckstein found that a hammerhead Rz cleaved a 19-mer oligoribonucleotide substrate 1,000 times more efficiently than a 985-nucleotide substrate containing the same target sequence. Taking various studies into account, Ellis and Rogers consider that the rate limiting step is likely to be either “finding the target” or “refolding the substrate and Rz to form the active hammerhead complex.”

Furthermore, in this study, the products of cleavage have been directly detected in vivo by an RNase protection assay. Usually, with less sensitive methods (as Northern blot for instance), these fragments could not be detected and the effect due to the Rz was only evaluated by the degree of reduction of the corresponding target. Moreover, in most cases, the Rz inhibition could also be due to its antisense effect as demonstrated with mutations of its catalytic domain. On the other hand, in vivo cleavage products have been described in a few other Rz studies: Steinecke et al used RNase protection to prove hydrolytic cleavage by their Rz, but only the 5' cleavage product was detected, probably because it has a cap structure and was better protected against 5'→3' exonuclease activity than the unprotected 5' end of the 3' cleavage product. Dropulic et al used PCR to quantify cleaved and uncleaved RNAs in Rz-containing cells infected with HIV-1. In our RNase protection experiments, in vivo cleavage was clearly demonstrated and corresponded to the protection of two RNA fragments of 75 nucleotides (pP2) and 199 nucleotides (pP1) exactly as predicted. In addition, a larger protected fragment of approximately 78 to 80 nucleotides was observed on in vitro and in vivo cleavages. It probably could be due to an unspecified hybridization of the probe, because it was also observed in untransfected UAC cells and in clones with low Rz activity (R3 and R9). As suggested above, the decrease of IL-6 production and of its mRNA expression correlates with the level of the IL-6 Rz catalytic activity; however, an additional antisense effect cannot be excluded.

Southern blot analysis showed that the instability of the pRe/CMV constructs observed in our transfected clones is
Fig 7. Rz-mediated catalytic activity. (A) The relative position of the Rz cleavage site within the IL-6 mRNA and the size of the expected protected fragments are indicated. (B) RNase protection of the Rz-treated IL-6 mRNA. Total RNA from stable transfectants was isolated as described in Materials and Methods (PCR reaction) and 50 μg was used for mapping by the RNase protection assay with a 327-nucleotide RNA probe (500,000 cpm). (Left) Lanes 1 and 2, in vitro cleavage reactions without and with Rz, respectively (excess 4x Rz, 10 minutes, 50°C). Lane 3, uninduced untransfected UAC. Lanes 4 and 5, RNA from untransfected UAC induced overnight with 3 and 10 ng/mL TNF-α, respectively. Lane 6, uninduced transfected R16 clone after 6.5 months of culture. Lane 7, induced transfected R16 clone after 6.5 months of culture. Lane 8, induced transfected R16 clone after 4 months of culture. Lane 9, Hpa II-digested PBR 322–molecular weight marker as described in Fig 2. Some of the molecular weight markers are indicated on the right. The bands indicated on the left correspond to the protected 3’ (pP1) and 5’ (pP2) cleavage fragments of the IL-6 mRNA. Exposition times are 1 night and 1 week for lanes 1, 2, 9, and 3-8, respectively. (Right) This RNase assay shows results with the four different clones. Lane 1, Hpa II-digested PBR 322–molecular weight marker as described in Fig 2. Lane 2, uninduced untransfected UAC. Lane 3, RNA from untransfected UAC induced 10 hours with 10 ng/mL TNF-α. Lanes 4 and 5, induced transfected clones R3 and R9, respectively. Lanes 6 and 7, induced transfected clones R16 and R21, respectively. Lanes 8 and 9, in vitro cleavage reactions without and with Rz, respectively (excess 4x Rz, 10 minutes, 50°C). All these assays were performed with RNAs extracted at 4 months of culture. The bands indicated on the right correspond to the protected 3’ (pP1) and 5’ (pP2) cleavage fragments of the IL-6 mRNA.
probably due to its episomal form in UAC cells (data not shown). The reasons for this episomal form remain unclear. The plasmid was poorly integrated in genomic DNA, perhaps because it had not been linearized before transfection. The episomal form was shown to be unreplicated and was thus probably progressively diluted.

This study shows that the Rz-mediated inhibition of IL-6 expression was more efficient when IL-6/mRNA to Rz ratios were low. Therefore, to prevent IL-6 overexpression in some pathologic situations, ie, myeloma and Kaposi’s sarcoma, other techniques might be explored. Besides the use of eukaryotic mammalian expression vectors, it should also be possible to use chemically modified Rzs with enhanced stability, which are then introduced into cells, by high-performance transfection techniques.

The in vivo activity of this IL-6 Rz should stimulate further work to elucidate the role of IL-6 in various pathologies. Similar approaches could be applied, in principle, to many other cytokines or growth factors.

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