0026-895X/96/010058-05$3.00/0

MOLECULAR PHARMACOLOGY, 49:58–62 (1996).

Inhibition of Human Interferon-γ Biosynthesis by an Antisense RNA-Expressing Vector

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Received March 28, 1995; Accepted October 5, 1995

SUMMARY

We applied antisense RNA technology for reducing the level of human IFN-γ (HuIFN-γ) expression. An antisense RNA vector containing the full-length HuIFN-γ cDNA in the opposite orientation was electroporated into cells constitutively producing very high levels of the cytokine. Approximately 53% of the resulting clones exhibited a specific HuIFN-γ inhibition of an average of 95.5%. The results of reverse transcription-polymerase chain reaction and Northern blot analyses revealed that the antisense effect originated from a specific reduction of the targeted mRNA caused by antisense RNA expression. This very effective antisense RNA strategy can have possible therapeutic applications in treating diseases where HuIFN-γ is known to play a negative role, such as in certain autoimmune diseases.

IFN-γ, which is produced by activated T lymphocytes and NK cells, is a pleiotropic protein. It not only plays a role in host defense against viral infections but also has antiproliferative effects and is involved in immunoregulatory functions. IFN-γ has been identified as the principal macrophage-activating factor regulating the differentiation of monocytes and inducing different immunological functions assigned to macrophages, such as Ag presentation, phagocytosis, and antitumoral properties. IFN-γ can inhibit or stimulate the antibody secretion of B lymphocytes and has pleiotropic effects on T cells. The biological actions of HuIFN-γ are reviewed in two recent articles (1, 2).

In contrast to these vitally important functions, IFN-γ plays a critical role in the pathology of a number of autoimmune diseases, including systemic lupus erythematosus (3), multiple sclerosis (4, 5), rheumatoid arthritis (6), and type 1 diabetes mellitus (7). A negative role for IFN-γ has also been demonstrated in graft-versus-host reactions (8), delayed-type hypersensitivity (9), allograft rejections (10), and chronic inflammations (11). IFN-γ enhances major cell-mediated immune responses by inducing major histocompatibility class II Ag expression on various cell types, including monocytes (12), macrophages (13), B cells (13), endothelial cells (14), and astrocytes (15), enabling these cells to present Ag to T cells. The inflammatory response is further boosted by its ability to induce immune effector cells, such as monocytes, macrophages, or NK cells, to phagocytosis, cytotoxicity, and release of reactive oxygen or nitrogen intermediates and inflammatory mediators such as TNF-α or interleukin-1 (16). Blockade of the production of IFN-γ can be directly anti-inflammatory in these cases, and several studies support the therapeutic effectiveness of IFN-γ inhibitors. Monoclonal (10) or polyclonal (8) antibodies to IFN-γ or to the secreted form of the IFN-γ receptor (17) have been used as IFN-γ antagonists.

In a previous report (18), we demonstrated the effectiveness of antisense oligodeoxynucleotide strategy in inhibiting the HuIFN-γ production by activated lymphocytes. An inhibitory effect of up to 90% has been achieved with a 16-mer spanning the translation initiation codon. In this study, HuIFN-γ producing cells were stably transfected with an antisense RNA expression vector. Antisense RNA technology has been used before to inhibit the cytokine IFN-β (19) and TNF-α (20, 21) expression, but to our knowledge, this is the first report in which this strategy has been applied for reduction of HuIFN-γ levels.

Materials and Methods

Construction of antisense SVXIFN. HuIFN-γ cDNA was inserted in the opposite direction in the *Bam*HI cloning site of the murine retrovirus shuttle vector pZIP-Neo SV(X) (22). To limit the size of the 1768-bp HuIFN-γ cDNA-containing *Sau*3AI fragment of the plasmid pUC18.gif.Hu, a 521-bp *Kpn*I/*Eco*RI fragment from pGV462 (23), containing several *Sau*3AI restriction sites, was inserted in the plasmid just upstream of the HuIFN-γ cDNA (this construct was called WEG). The HuIFN-γ cDNA could then be isolated as a 854-bp *Sau*3AI fragment and was inserted into the *Bam*HI cloning site of pZIP-Neo SV(X). The antisense orientation of the

We are grateful to the Nationaal Fonds voor Wetenschappelijk Onderzoek for research fellowships (C.M.A.B.).

ABBREVIATIONS: HuIFN-γ, human IFN-γ; IFN, interferon; NK, natural killer; Ag, antigen; TNF, tumor necrosis factor; bp, basepair; CHO, Chinese hamster ovary; DHFR, dihydrofolate reductase; RT, reverse transcription; PCR, polymerase chain reaction; nt, nucleotide.

insert was confirmed by the *Sma*I restriction pattern, and the construct was called antisense SVXIFN.

Cell lines and culture conditions. 12C MTX is an established cell line obtained by stable cotransfection of CHO DHFR$^-$ cells with a human HuIFN-γ cDNA and a murine DHFR cDNA expression vector. The HuIFN-γ expression of a selected clone (12C) was amplified by applying methotrexate at escalating concentrations; the resulting clone produces HuIFN-γ at ~30,000 IU/ml (24).

Cells were grown in minimum essential medium supplemented with 2 mM L-glutamine, 0.005% (w/v) gentamycin, 0.011% (w/v) $NaHCO_3$, pH 7.0, and 10% (v/v) heat-inactivated (56°, 30 min) fetal calf serum at 37° in a 5% (v/v) CO_2 humidified atmosphere and maintained in the same medium but supplemented with 2% fetal calf serum instead of 10%. Media and supplements were purchased from GIBCO (Paisley, UK).

Cell transfection and selection of stable transfectants. 12C MTX cells were transfected with plasmid DNA by electroporation (25). Nonlinearized plasmid DNA was electroporated into 6×10^5 cells suspended in 600 μl phosphate buffered saline using a Bio-Rad gene pulser (Bio-Rad, Hercules, CA) set at 0.2 kV and 960 μF. Cells were selected 1 week later by supplementing the standard medium with 480 μg/ml G418 (Geneticin, G418 sulfate, GIBCO). Stable cell clones were obtained after 2–3 weeks' growth in the drug-containing medium.

HuIFN-γ levels in cell supernatants were assayed with HuIFN-γ-specific sandwich enzyme-linked immunosorbent assay, as described previously (18). Total RNA was isolated by acid guanidinium thiocyanate/phenol/chloroform extraction (26).

RT-PCR amplification. The GeneAmpRNA PCR kit (Perkin Elmer Cetus, Norwalk, CT) was used for PCR amplification under the conditions recommended by the manufacturer with minor modifications. One microgram of total RNA was reverse-transcribed: 50 min at 37°, followed by heat-inactivation of the enzyme at 99° for 5 min. The PCR amplification reaction was carried out in a Techne (Cambridge, UK) thermocycler for 20 cycles at 95° for 1 min, 55° for 1 min, and 72° for 2 min starting from the total RT product for analysis of HuIFN-γ and actin mRNA levels. In the case of antisense RNA amplification, 35 cycles were carried out at 95° for 1 min, 60° for 1 min, and 72° for 2 min on an 80th of the RT product. The higher temperature of primer annealing (60°) and the lower amount of starting material were necessary to avoid nonspecific PCR products.

For HuIFN-γ mRNA amplification, a 5′ primer, 5′-GACGAATTCATGCAGGACCCATATG-3′ (with the same polarity as nt 70–91 (27)), and a 3′ primer, 5′-ATAAGAAGCTTTCAAATATTGCAGGCAGGAC-3′, complementary to a sequence at the very beginning of the 3′ untranslated region (nt 507–527), were used. Each primer also contains a restriction enzyme site for use in other experiments. The 5′ primer 5′-GTGGGGCGCCCCAGGCACCA-3′ and 3′ primer 5′-GTCCTTAATGTCACGCACGATTTC-3′, with the same sense as nt 103–122 (28) and complementary to nt 619–641, respectively, were used for amplification of β-actin; the primers 5′-GCTTTTCGAAGTCATCTCGTT-3′, complementary to nt 332–352 of the *HuIFN*-γ gene, and 5′-AGGGAAGTTTACAAGGG-3′, with the same sense as a sequence of the vector pZIP-Neo SV(X) starting at ~50 nt distant from the HuIFN-γ antisense insert for amplification of antisense RNA.

Northern and Southern blotting. Twenty micrograms of total RNA or one tenth of the obtained PCR product were electrophoresed on a 0.8%/2.2 M agarose/formaldehyde or 1% agarose gel, respectively. Samples were transferred to a nylon membrane by vacuum blotting (VacuGene XL, Pharmacia, Uppsala, Sweden), and hybridization was carried out with biotin-labeled probes using the Gene Images Nonisotopic Nucleic Acid Detection Kit (USB, Cleveland, OH). The probes were biotin labeled by means of a PCR reaction with the appropriate primers and biotin-21-dUTP (Clontech, Palo Alto, CA) on the plasmid WEG (see Construction of antisense SVXIFN) for detection of HuIFN-γ mRNA or antisense RNA or by means of a PCR reaction on cDNA obtained by RT-PCR on total RNA with the actin primers for detection of actin mRNA. Prehybridization, hybridization, washing, and subsequent chemiluminescent detection were performed according to the instructions of the manufacturer. Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate (Tropix, Bedford, MA) was used as the substrate for alkaline phosphatase. Luminescent filters were exposed to X-ray films (Fuji, Tokyo, Japan).

Results

Expression of antisense RNA in 12C MTX cells. The entire coding sequence flanked by ~55 bp of the 5′and 286 bp of the 3′ untranslated region of the *HuIFN*-γ gene was inserted into the retrovirus vector pZIP-Neo SV(X) in an antisense orientation relative to the Moloney murine leukemia virus long terminal repeat promotor; the construct was called antisense SVXIFN.

The 12C MTX cell line constitutively produces HuIFN-γ with very high yields at ~30,000 IU/ml. Cells were transfected with 3.9, 10, 15, or 20 μg antisense SVXIFN; 17 stable transfectants were isolated on the basis of colony formation in 480 μg/ml G418.

Effect of antisense RNA on HuIFN-γ expression. The level of HuIFN-γ in the transfected cells was measured by a HuIFN-γ-specific enzyme-linked immunosorbent assay and compared with HuIFN-γ yields from the same amount of nontransfected 12C MTX cells. The results are shown in Fig. 1. The 17 transfectants were divided into two groups: eight clones showing no change in HuIFN-γ levels compared with nontransfected 12C MTX cells, and nine clones exhibiting a reduction of an average of 95.5%.

As an additional control, 12C MTX cells were transfected with the pZIP-Neo SV(X) vector without insert (10 or 20 μg). HuIFN-γ levels of 25 of the 28 obtained transfectants were identical to levels of nontransfected 12C MTX cells; however, the HuIFN-γ expression of three clones (11%) was reduced by 99% compared with nontransfected cells. This reduction was probably the result of integration of the plasmid at a site interrupting the sequence encoding or controlling the

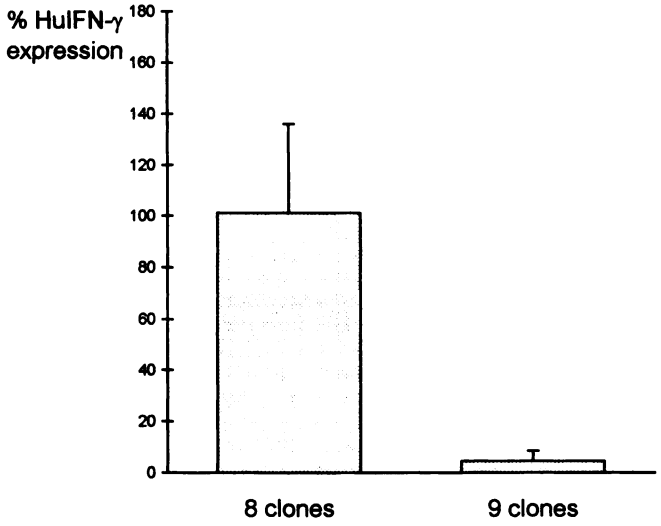

Fig. 1. Effect on the HuIFN-γ expression of antisense SVXIFN transfection in 12C MTX cells. 12C MTX cells were transfected with 3.9, 10, 15, or 20 μg antisense SVXIFN; HuIFN-γ levels of the resultant transfectants were detected with a HuIFN-γ-specific enzyme-linked immunosorbent assay and compared with HuIFN-γ yields from the same amount of nontransfected 12C MTX cells.

HuIFN-γ protein; experiments are currently performed to confirm this hypothesis. The disparity between the percentage of antisense-transfected 12C MTX cells and the vector-alone control cells exhibiting reduced HuIFN-γ expression allows a tentative conclusion that antisense RNA mediates HuIFN-γ inhibition.

RNA expression. To investigate the mechanism of inhibition by the antisense vector, HuIFN-γ mRNA accumulation was determined by two independent methods and antisense RNA levels were analyzed using RT-PCR amplification.

First, we analyzed HuIFN-γ mRNA levels by performing an RT-PCR reaction of 20 cycles on equal amounts of RNA isolated from nontransfected 12C MTX cells, nontransfected CHO cells, and four different clones of antisense plasmid transfected cells. Fig. 2A shows that there is a good correlation between the amount of the obtained PCR product on agarose gel and the percentage inhibition of the HuIFN-γ production of an individual clone. RNA extracted from nontransfected 12C MTX cells and from clones that showed no inhibition of HuIFN-γ production showed equally intense bands (data not shown). In contrast, clones 3.9B1 (which showed 90% inhibition of HuIFN-γ production) and 15B3 (95% inhibition) yielded correspondingly less PCR product (*lanes 2 and 3*), whereas no band was detectable for clone 15C1 (99–100% inhibition) (*lane 4*). As a negative control, RT-PCR was performed on RNA from CHO cells, which obviously do not express HuIFN-γ mRNA (*lane 5*).

As a control for the specificity of HuIFN-γ antisense RNA and for the integrity of the extracted RNA, we performed an RT-PCR with actin primers on the same samples. Fig. 2B shows that actin mRNA levels are not affected by HuIFN-γ antisense RNA transfection.

A linear response between the amount of RNA input and the amount of RT-PCR product was shown to occur in the range of 15–25 cycles. Therefore, RT-PCR reactions of 20 cycles were performed to compare RNA levels of different cell lines.

Northern analysis was used as a second method to identify endogenous HuIFN-γ mRNA. The biotin-labeled HuIFN-γ cDNA hybridized to transcripts of ~6.5 kb (Fig. 3A). mRNA accumulation was reduced in the clone 3.9B1 (exhibiting a 90% inhibition of HuIFN-γ production) (*lane 2*) compared with nontransfected 12C MTX cells. The endogenous transcript was not detectable in the clone 15C1 (*lane 3*), which showed 99–100% inhibition of HuIFN-γ production. No additional bands, corresponding to the expression of antisense RNA, could be detected.

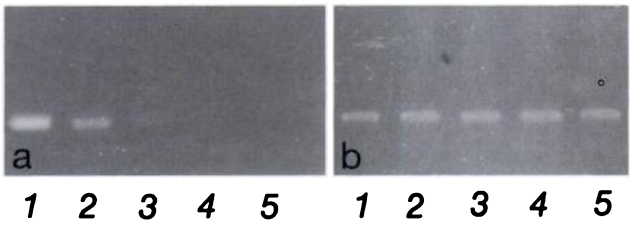

Fig. 2. RT-PCR on total RNA isolated from antisense SVXIFN transfected 12C MTX cells. One microgram of total RNA isolated from different cell clones was used as a template for RT-PCR analyses. Specific HuIFN-γ (A) or actin (B) primers were used yielding a 480- or 540-bp PCR product, respectively. The PCR-amplified products were separated by electrophoresis in a 1.5% agarose gel in Tris/acetate/EDTA buffer. *Lane 1*, nontransfected 12C MTX cells; *lane 2*, clone 3.9B1 (90% inhibition of HuIFN-γ production); *lane 3*, clone 15B3 (95% inhibition); *lane 4*, clone 15C1 (99–100% inhibition); and *lane 5*, blank CHO cells.

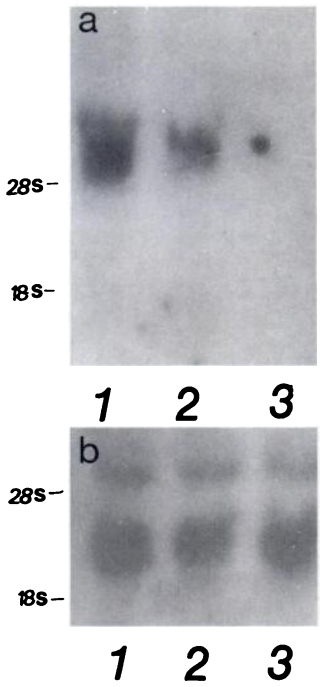

Fig. 3. Analysis of total RNA from antisense SVXIFN transfected 12C MTX cells by Northern blotting. Twenty micrograms of total RNA was separated on a 0.8%/2.2 M agarose/formaldehyde gel, transferred to a nylon membrane, and hybridized with biotin-labeled HuIFN-γ (A) or actin (B) cDNA probes using the Gene Images Nonisotopic Nucleic Acid Detection Kit (USB). *Lane 1*, nontransfected 12C MTX cells; *lane 2*, clone 3.9B1; and *lane 3*, clone 15C1.

Equal quantities of the actin mRNA were observed with biotin-labeled actin cDNA probes (Fig. 3B), showing again that the observed effect of antisense SVXIFN transfection is specific.

Fig. 4 shows the results of RT-PCR analysis of antisense RNA. A faint band can be seen for the clones 3.9B1, 15B3, and 15C1 (*lanes 1*, *2*, and *3*, respectively), which reduced the HuIFN-γ expression by ≥90%, whereas no PCR product was detectable for clones that did not inhibit the HuIFN-γ expression. When PCR amplification was carried out omitting the RT step, no bands were visible (results not shown), proving that the RNA samples were not contaminated with DNA.

Discussion

IFN-γ, produced by activated T lymphocytes and NK cells, is one of the most important cytokines. In addition to its

Fig. 4. Antisense RNA analysis of antisense SVXIFN transfected 12C MTX cells using RT-PCR amplification followed by Southern hybridization. RT-PCR amplification on total RNA from different clones was carried out to detect antisense RNA. PCR products were electrophoresed on a 1% agarose gel, transferred to a nylon membrane, and hybridized to a biotin-labeled HuIFN-γ cDNA probe. *Lane 1*, clone 3.9B1; *lane 2*, clone 15B3; *lane 3*, clone 15C1; and *lanes 4 and 5*, two clones exhibiting no inhibition of HuIFN-γ expression.

antiviral and antiproliferative properties, it has important immunoregulatory functions. But IFN-γ is also believed to play a negative role in diseases where the immune system is deregulated. Several clinical studies indicate a therapeutic value of IFN-γ antagonists in the treatment of various autoimmune diseases. We report the successful inhibition of the HuIFN-γ expression with the use of the antisense RNA technology. In a previous report (18), we investigated 12 antisense oligodeoxynucleotides complementary to various sequences of the HuIFN-γ gene for their ability to decrease the HuIFN-γ production of activated lymphocytes. A 16-mer, specific for a sequence including the translation initiation codon, was found to be most effective, demonstrating an inhibition of up to 90% when added in separate doses and at a total concentration of 200 μM. Three other 16-mers, complementary to a sequence in the 5′ noncoding region, in the coding region, or at the donor splice junction of the third intron respectively, were inhibitory to a lesser extent (40–50%).

In the present study, an antisense recombinant plasmid (3.9, 10, 15, or 20 μg) was electroporated into 6×10^5 12C MTX cells. Approximately 53% of all obtained clones exhibited a ≥90% reduction of the HuIFN-γ production that was constitutive even when cells were grown in the absence of G418 for longer periods. This result and the fact that nontransfected 12C MTX cells produce yields as high as 30,000 IU/ml of HuIFN-γ demonstrate that this antisense approach is very efficient.

To determine the mechanism by which the antisense RNA inhibits the expression of mRNA, the accumulation of target HuIFN-γ mRNA and antisense RNA in the cell was investigated. The results of an RT-PCR reaction on total RNA isolated from different clones of transfectants and from nontransfected 12C MTX cells revealed that the inhibition of the HuIFN-γ expression originated in the specific reduction of the target mRNA caused by the expression of antisense RNA in these clones. A close correlation was observed among the reduction of the level of the transcript, the encoded protein, and the expression of antisense RNA. Antisense-transfected clones that did not express antisense RNA produced the same amount of HuIFN-γ as nontransfected 12C MTX cells. Clones exhibiting 90%, 95%, and 99% inhibition, respectively, yielded gradually less HuIFN-γ/PCR product and obviously did express antisense RNA.

The other method used to estimate mRNA levels, Northern blotting, gave similar results. mRNA accumulation was reduced in transfectants exhibiting a reduction in HuIFN-γ levels and showing antisense RNA expression, whereas no transcript was detectable when Northern analysis was performed on RNA extracted from clones that showed nearly complete protein inhibition and did express the antisense RNA. The biotin-labeled HuIFN-γ cDNA hybridized to transcripts of ~6.5 kb. The length of the message is not surprisingly long because the HuIFN-γ expression of the original 12C clone was amplified with methotrexate for long periods of time, which may result in the incorporation of multiple copies of the HuIFN-γ gene in the genome of the CHO cells, which can be transcribed polycistronically. This situation also occurs in nature, where it has been shown that certain genes of several eukaryotic organisms can be transcribed as polycistronic mRNAs (29, 30).

Because no reduction in the level of actin transcripts in transfected cells could be observed, we can conclude that the antisense effect is specific.

There are several possible mechanisms by which antisense RNA can block expression of the targeted gene. Hybridization of the antisense RNA with the sense mRNA results in the formation of double-stranded RNA. These duplexes can either block the translocation of the mRNA from the nucleus to the cytoplasm (31, 32), impair mRNA processing (33–35), or cause it to be rapidly degraded (31, 34). From the presented results, it is not possible to distinguish among these mechanisms.

In the present report, we used antisense RNA technology to reduce the level of HuIFN-γ expression of cells producing high IFN-γ levels. Transfection with a vector that allows constitutive expression of full-length antisense transcripts was demonstrated to be very effective.

An inhibition of the HuIFN-γ production of an average of 95.5% was achieved in ~53% of the obtained transfectants. This antisense RNA expression system thus seems to be more effective than antisense oligodeoxynucleotides in inhibiting protein synthesis. The potency of inhibiting the expression of one specific protein will also help to delineate the specific role of HuIFN-γ in the complex cytokine network. This antisense RNA strategy can have potential applications in the treatment of diseases where HuIFN-γ is shown to mediate the pathology, such as in a number of autoimmune diseases. The use of such gene therapy allows stable, complete inhibition of the HuIFN-γ expression in a very early stage and in a 100% specific manner.

Acknowledgments

We wish to thank Dr. A. G. Morris (University of Warwick, Department of Biological Sciences, Coventry, UK) for his kind gift of 12C MTX cells.

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