Expression of interferon-γ is one of the most important factors influencing both the progression of chronic HCV infections, as well as the treatment results. Currently, the only therapy officially registered in the chronic hepatitis C includes interferon-α and ribavirin administration. Implementation of interferon-α in hepatitis C treatment as a monotherapy had positive results in only 12-20% of patients. The use of ribavirin, a synthetic nucleoside analogue, in combination with interferon-α has increased the therapy efficiency to more than 40%, and to over 60% when it is administered with pegylated form of IFN-α (1, 2). Interestingly, ribavirin itself is almost completely ineffective against HCV and does not lead to sustained elimination of HCV viremia when used alone. Therefore, it has been suggested that there is an indirect mechanism of ribavirin beneficial action in hepatitis C (3, 4). Perhaps the main activity of ribavirin leading to HCV elimination is connected with its antiinflammatory action. The combined therapy based on interferon-α and ribavirin has become a gold standard in the chronic hepatitis C, and it is the only disease where those drugs are used in combination.

Despite the intensive studies on the HCV-specific forms of therapy such as HCV polymerase or protease inhibitors, as well as antisense and RNAi technologies, a traditional IFN-α and RIBA-based therapy seems to have long future (5), even though there are still many controversies concerning the mechanisms of those drugs action. They both have direct antiviral activities but also at least a part of their action is mediated by indirect influence on immunological system and expression of many immunoregulatory genes, including interferon-γ (6-8). The aim of the study was to investigate the direct influence of interferon-α and ribavirin on the transcriptional activities of IFN-γ and its receptor in the in vitro model.
EXPERIMENTAL

Chemicals

Interferon-α (2.00 x 10^6 IU/mL), ribavirin, concanavalin A, cyclosporin, 0.4% trypan blue solution, were purchased from Sigma-Aldrich. Dexamethasone (Dexaven) was purchased from Jelfa (Poland). All chemicals used for Jurkat in vitro cell cultivation (RPMI-1640, FBS, PBS, gentamycin) were purchased from PAA.

Cell cultures conditions and stimulation

Experiments were carried out on the in vitro cultures of Jurkat Clone E6-1 cell line (ATCC: TIB-152). Cell cultures were routinely propagated in a humidified incubator Hera-Cell (Heraeus), at 37°C, in 5% CO₂/95% air atmosphere. Cells were grown in RPMI-1640 medium supplemented with 10% FBS and 5 mg/mL gentamycin. Twenty hours before treatment with the studied chemicals, cells were stimulated with concanavalin A (10 μg/mL) for 1 h to express high levels of interferon-γ, according to literature data. After the stimulation, cells were centrifuged, washed in PBS, and transferred to 24-well culture plates. Treatment with interferon-α and ribavirin was carried out for 6 h. IFN-α was used at 100, 500, 1000, and 2000 IU/mL; RIBA was used at 60, 120, 240, and 480 IU/mL. Both drugs were also used in combination in the whole range of concentrations. After the treatment cells were centrifuged, washed in PBS, and used for RNA extraction. Additional experiments using reference chemicals were carried out to confirm the specificity and reliability of the methodology. The following chemicals were used: dexamethasone (0.5 μM/L), cyclosporin (3 μM/L), and concanavalin A (5 μg/mL).

Cytoxicity assay

In order to rule out non-specific cytoxicity of interferon-α, ribavirin or other chemicals used, trypan blue exclusion test was used. Cell cultures were treated with the drugs in the same manner as in all other experiments. After treatment cells were centrifuged, washed in PBS, mixed with 0.4% trypan blue solution in equal volumes, and transferred to a hemacytometric Neubaeur’s chamber. The numbers of dead/alive cells were counted using a light inverted microscope (Nikon TSM).

RNA extraction

Total RNA was extracted by phenol-chloroform method using TRI-Reagent (Sigma-Aldrich) according to manufacturer’s protocol. The RNA concentration was determined spectrophotometrically by measuring the absorbance at 260 nm. RNA quality was estimated by 2% agarose gel electrophoresis with ethidium bromide staining.

Real-Time RT-PCR assay

Levels of mRNA of the interferon-γ, IFN-γ receptor subunits (IFNgR1, IFNgR2), and β-actin were determined by SYBR Green Real-Time RT-PCR assay. RT-PCR reaction tubes contained 12.5 μL of 2x QuantiTect SYBR Green RT-PCR Master Mix (containing HotStart Taq DNA Polymerase, QuantiTect SYBR Green RT-PCR Buffer with fluorescent dyes SYBR Green I and ROX, and dNTP mix), 0.5 μL of reverse transcriptase mix (OmniScript and Sensiscript), 0.3 μM of each sense and antisense primer, 0.4 μg of unknown RNA template, and water to a total volume of 25 μL. All reagents were purchased from Qiagen. One-step Real-Time RT-PCR assay was carried out using ABI PRISM 7000 Sequence Detector (Applera). The thermal profile was 50°C for 30 min (reverse transcription), and 95°C for 15 min, 40 two-step cycles of 94°C for 15 s and 60°C for 30 s, and 72°C for 10 min (Real-Time PCR), followed by a dissociation protocol (60-95°C; 30 min).

Sequence-specific PCR primers for mRNA of IFN-γ and its receptor subunits (IFNgR1 and IFNgR2) were designed using computer software Primer Express v.1.0 ABI PRISM (Applera). The following oligonucleotide 5’ and 3’ primer sequences were used: β-actin – sense: 5’TCA CCC ACA CTG TGC CCA TCT AGC A3’; antisense: 5’CAG CGG AAC CGC TCA TTG CCA ATG G3’; (amplification product 295 bp); IFN-γ – sense: 5’TGA ACT CAT CCA AGT GAT GGC TGA ACT ATG GCTC3’; antisense: 5’CAGCGAGGACACCATTACTGGGAT-GCTC3’; (amplification product: 115 bp); IFNgR1 – sense: 5’ATA CCG AAG ACA ATC CAG GAA AAG TGG AAC A3’; antisense: 5’GCC ATG CTC GGA GGA CAG CTC ACC AAA GGA TGA CG3’; anti- sense: 5’CAGCAGGAGACACCATTACTGGGATGCTC3’; (amplification product: 115 bp); IFNgR2 – sense: 5’ATA CCG AAG ACA ATC CAG GAA AAG TGG AAC A3’; antisense: 5’GCC ATG CTC GGA GGA CAG CTC ACC AAA GGA TGA CG3’; anti- sense: 5’CAGCAGGAGACACCATTACTGGGATGCTC3’; (amplification product: 151 bp).

The amplification products were separated on 8% polyacrylamide gels and visualized using silver staining. The specificity of PCR products was additionally confirmed by determination of dissociation curves assay run after every Real-Time RT-PCR amplification by measuring fluorescence intensity during temperature elevation between 60 and 95°C. All amplimers were sequenced with the use of ABI PRISM 377 plate sequencer (Applera), and Big Dye chemistry.
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Data interpretation and statistical analysis of the Real-Time RT-PCR results

To evaluate the changes in mRNA expression of the studied genes (interferon-γ, IFNgR1 and IFNgR2) ΔΔCt method of relative expression estimation was used. mRNA levels of the studied genes were normalized to endogenous control (β-actin mRNA), and the calibrators were the control (untreated) cultures. Presented values were the results of mathematical equation $2^{\Delta\Delta C_t}$ which indicates the increase or decrease in mRNA expression of certain gene compared with the control sample (untreated culture) after normalization to the expression of the housekeeping gene (β-actin). All the results are presented as the medians ± interquartile range of three independent experiments, each performed in triplicate. The quantitative data were compared by nonparametric Mann-Whitney U test. p < 0.05 was considered significant. All calculations were performed with Statistica Version 6.0 software.

RESULTS

Expression of interferon-γ and IFN-γ receptor subunits (IFNgR1 and IFNgR2) were determined at mRNA level by means of Real-Time RT-PCR in total RNA extracts obtained from the in vitro Jurkat cell line cultures. In all cultures (treated or and untreated) detectable levels of mRNA of endogenous control gene, β-actin, were present, which indicates the presence of high-yield, undegraded RNA extracts. β-Actin performed high and stable expression therefore its mRNA was used as an endogenous control in ΔΔCt method to evaluate the expression of IFN-γ and its receptor.

In order to rule out unspecific cytotoxicity of interferon-α, ribavirin, and other chemicals, a trypan blue exclusion test was used. Two highest concentrations of IFN-α (1000 and 2000 IU), and RIBA (240 and 480 µM) were used. Both drugs were also tested in combination at 2000 IU of IFN-α and 480 µM of RIBA. A percentage of dead cells was similar in all studied cultures (mean 11.7 ± 3%), as well as in the untreated cultures (mean 9.9 ± 1.3%) (Table 1). It indicates the lack of unspecific cytotoxicity of the studied drugs towards Jurkat cells. The reference chemicals did not influence cell viability in the studied cultures either, except for dexamethasone which has antiproliferative features and thus increased slightly the number of dead cells compared with the control cultures (18.5%).

The influence of interferon-α and ribavirin on the expression of interferon-γ and IFN-γ receptor was compared with the influence of chemicals known in the literature as stimulators (concanavalin A) or inhibitors (cyclosporin, dexamethasone) of interferon-γ expression in lymphocytes. The obtained results indicate that the chemicals used in the study effectively influenced expression of both interferon-γ, and its receptor subunits (IFNgR1 and IFNgR2) (Fig. 1). Dexamethasone (0.5 µM) decreased mRNA levels of interferon-γ to 22% of the expression measured in untreated cultures ($2^{\Delta\Delta C_t} = 0.22$), and interferon-γ receptor subunits, IFNgR1 and IFNgR2, to 40% ($2^{\Delta\Delta C_t} = 0.40$) and to 35% ($2^{\Delta\Delta C_t} = 0.35$), respectively. Cyclosporin (3 µM) decreased expression of interferon-γ to 19% of the expression measured in untreated cultures ($2^{\Delta\Delta C_t} = 0.19$), to 51% ($2^{\Delta\Delta C_t} = 0.51$) of IFNgR1, and to 26% ($2^{\Delta\Delta C_t} = 0.26$) of IFNgR2. Unlike, concanavalin A increased expression of interferon-γ ($2^{\Delta\Delta C_t} = 1.6$), IFN-γ receptor subunit 1 ($2^{\Delta\Delta C_t} = 1.02$), and subunit 2 ($2^{\Delta\Delta C_t} = 2.27$).

Treatment of Jurkat cell cultures with interferon-α and ribavirin at different concentrations...
revealed complex involvement of the studied drugs in the regulation of interferon-γ and its receptor expressions. Interferon-α induced expression of interferon-γ from 1.52 to 1.96-times when compared with the control (untreated) cell cultures (Fig. 2a). Generally, the higher concentration of IFN-α was used, the stronger expression of IFN-γ demonstrated. IFN-α also induced expression of IFN-γ receptor (Fig. 3a, 4a); both subunits performed enhanced expressions: IFNγR1 was 1.98–7.67-times higher, and IFNγR2 was 1.83–2.53-times higher. The same evaluation for cells treated with ribavirin showed contrary results. Ribavirin strongly inhibited expression of interferon-γ causing a decrease in IFN-γ levels to 71–84% and even to 2% (at 240 µM) of the levels observed in the untreated cell cultures (Fig. 2b). Ribavirin also decreased mRNA levels of IFNγR1 (by 14–59%) and IFNγR2 (by 19–71%) (Fig. 3b, 4b). The strongest inhibitory effects were observed at 240 µM concentration of ribavirin.

Combined treatment with IFN-α and ribavirin revealed a strong inhibitory effect of ribavirin itself on the expression of all three studied genes, IFN-γ, IFNγR1, and IFNγR2, in Jurkat cell cultures (Fig. 5 a-c). Cells were treated either with increasing concentrations of both drugs (100 U IFN/60 µM RIBA; 500/120; 1000/240; 2000/480) or with increasing concentrations of IFN-α and decreasing concentrations of ribavirin (100 U IFN/480 µM RIBA; 500/240; 1000/120; 2000/60). In both treatment patterns the expressions of the studied genes decreased compared with the control (untreated) cultures. In all cultures ribavirin performed strong inhibitory effect even at the lowest concentration while the contrary effect of interferon-α was almost completely diminished.

**DISCUSSION AND CONCLUSION**

Interferon-γ is one of the major cytokines involved in the regulation of immunological response in viral infections. It is produced by activated T lymphocytes and NK cells. The main action of this cytokine is the induction of cellular (Th1-type) response which contributes to successful elimination of viremia. To exert full biological activity interferon-γ requires a dimeric receptor composed of two subunits: R1 (α chain) and R2 (β chain). Therefore, expression of all three genes is important for complete evaluation of IFN-γ action (9-11).
Upregulation of IFN-γ includes secretion of IL-2 and IFN-γ-trating mononuclear cells (14). Many papers indicate that in peripheral blood (12, 13), and in liver-infil-

mechanisms of liver damage. Response but also mediating immunopathological

as the element of dominating Th1-like immune

(15, 16). Thus, IFN-γ plays a dual role in hepatitis C as the element of dominating Th1-like immune response but also mediating immunopathological mechanisms of liver damage.

Although well documented in vivo, direct influence of the IFN-α and ribavirin anti-HCV ther-

apy on IFN-γ expression has not yet been studied fully. Therefore, we performed an in vitro study on Jurkat cell line expressing IFN-γ after mitogenic stimulation. Our results confirmed stimulatory effect of IFN-α on the expressions of both IFN-γ, and the IFN-γ receptor. On the contrary, ribavirin performed strong reverse action causing inhibition of all three studied genes expressions. This inhibitory effect was demonstrated at all concentrations used in the experiments even in the presence of high concentrations of IFN-α. Expressions of interefron-

and its receptor increased slightly only when the highest concentration of IFN-α (2000 IU) and the lowest concentration of ribavirin (60 µM) were used. We excluded suppressive influence of the drugs on Jurkat cells proliferation thus eliminating the possible unspecific decrease in interferon-γ expression caused by cytotoxic or antiproliferative effect of ribavirin. It cannot be ruled out that antiproliferative influence may be diminished in this type of cell line because of its neoplastic features, however, Jurkat serves as a well-established model cell line for T lymphocyte studies in vitro.

Our results are in concordance with papers indicating that during the anti-HCV therapy expres-
sion of interferon-γ in vivo changes significantly. Bergamini and co-workers (17) found that during the IFN-α and ribavirin therapy levels of IFN-γ mRNA decrease. The authors suggest that the decrease in IFN-γ expression is caused by ribavirin, however, they presented only indirect results conducted on peripheral lymphocytes from HCV-infected patients. Our results for the first time directly support this hypothesis. Results of Barnes et al. (18) suggest that both interferon-α, and ribavin exert their biological activities through the influence on the expression of certain cytokines. They found that in vitro stimulation of dendritic cells with IFN-α or ribavirin resulted in the enhanced production of IL-12, and TNF-α caused by IFN-α, but the suppression of the expression of both cytokines by RIBA. The inhibitory effect of ribavirin occurred at the mRNA level. This inhibition was postulated by authors to explain the antiinflammatory action of ribavirin in the liver. Shiffman et al. (19) found that interferon-α stimulated production of typical Th1 cytokines IL-2, and IFN-γ, as well as TNF-α by 1.9-8.3-fold in T lymphocytes after stimulation with phytohemaglutinin. Ribavirin was shown to have no impact on the expression of the cytokines. Both drugs also performed suppressive influence on lymphocyte proliferation. On the other hand, there are studies suggesting that ribavirin potentiates the anti-HCV activity of interferon-α by up-modulating expression of T cell cytokines, such as IL-2 and
IFN-γ (8, 20, 21). Ning and co-workers (22) reported that ribavirin inhibited Th2 response and reduced levels of proinflammatory cytokines (TNF-α, IL-1) although it did not influence IFN-γ production. Zhang and collaborators (23) indicate that the possible mechanism of RIBA’s beneficial role in chronic hepatitis C is a result of its influence on the expression of certain IFN-α-stimulated genes (ISG) which was observed in respiratory syncytial virus (RSV)-infected cells. They proved that ribavirin enhanced expression of those genes by stimulatory effect on binding of transcriptional factors to ISRE sequences (interferon-stimulated response element) in their regulatory DNA regions contributing to antiviral response. The authors suggest that ribavirin may act as the agent that sensitizes cells to interferon-α through activating ISRE-dependent signalling. Recent papers suggest that elevated levels of interferon-γ in chronic hepatitis C have rather disadvantageous significance and that is indirectly connected with HCV viremia. Larrea et al. (24) proved by both in vitro and in vivo analyzes that HCV replication inhibits molecular signalling pathways of interferon-α by interfering in the activity of cytoplasmic adaptor proteins (STATs — signal transducers and activators of transcription) which are the main element of interferon signalling. At the same time, HCV does not influence similar STATs signalling of interferon-
The overall effect is a decreased IFN-α responsiveness of infected cells and undisturbed IFN-γ signalling which, together with high expression of IFN-γ, results in the progression of necroinflammatory processes. In this regard, decreasing expression of the cytokine during IFN-α/RIBA therapy should be beneficial. Earlier report of Bergamini et al. (17) suggesting such hypothesis was confirmed by us in the direct in vitro analysis described in the following paper. In conclusion, we suggest that the inhibition of interferon-γ expression and signalling by ribavirin seems to be an important mechanism of therapeutic effect of the combined treatment of chronic hepatitis C patients with interferon-α and ribavirin.

REFERENCES


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