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Inhibition of coxsackievirus B3 carrier state infection of cultured human myocardial fibroblasts by ribavirin and human natural interferon-α

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Abstract

As enterovirus infections of the heart cause myocarditis and eventually congestive heart failure, the antiviral activity of ribavirin was studied in coxsackie virus B3 (CVB3)-infected carrier cultures of human myocardial fibroblasts. Cultures were infected 7 days before application of ribavirin and effects were evaluated over a period of 16 days by plaque assays and in situ hybridization. Compared to the low antiviral activity in HeLa cells, ribavirin was highly active in reducing infectious virus yields in human myocardial fibroblasts, for example, to 2.0×10^3 pfu/ml with 25 μg/ml and to 1.3×10^2 pfu/ml with 50 μg/ml (4.3×10^4 pfu/ml in infected controls). Moreover, 100 μg ribavirin/ml completely suppressed infectious virus progeny in two of three cultures, and reduced the number of infected cells from 14.3 to 0.3% as determined by in situ hybridization, whereas up to 3200 μg ribavirin/ml did not result in a significant cytotoxic effect. Interaction with interferon-α (IFN-α) was additive to slightly synergistic in reducing the number of infected cells and virus yields. In conclusion, our results suggest a cell-specific high activity of ribavirin in human myocardial fibroblasts and indicate the importance of using organ-specific cells for testing antiviral agents in myocarditis. Furthermore, the usefulness of in situ hybridization for determining the long term effects of antivirals in carrier state cell cultures was demonstrated. © 1997 Elsevier Science B.V.

Keywords: Ribavirin; Interferon-α; Myocarditis; Myocardial fibroblasts; In situ hybridization

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1. Introduction

Many enterovirus infections are probably subclinical, but infections of the heart, especially with the cardiotropic coxsackie viruses of group B (CVB, types 1–6) may cause serious diseases, for example myocarditis with life-threatening arrhythmias (Abelmann, 1973, Kandolf and Hofschneider, 1989; Muir, 1993; Why et al., 1994). Chronic enterovirus infections of the heart may result in congestive heart failure with the clinical picture of dilated cardiomyopathy and the only remedy then may be heart transplantation (Why et al., 1994; Figulla et al., 1995). Autoimmunologic pathogenesis was suspected in myocarditis and dilated cardiomyopathy previously (Huber et al., 1985; Woodruff and Woodruff, 1974), whereas newer studies favour direct myocardial damage by enterovirus replication in myocytes and myocardial fibroblasts (Koide et al., 1992; Kandolf and Hofschneider, 1989; Klingel et al., 1992; McManus and Kandolf, 1991; McManus et al., 1993). Moreover, three controlled clinical trials of immunosuppressive therapy in myocarditis and dilated cardiomyopathy, which were initiated under the concept of immunopathogenesis, were unable to detect positive effects (Latham et al., 1989; Parillo et al., 1989; Mason et al., 1995). Furthermore, a prospective study on the natural course of myocarditis and dilated cardiomyopathy confirmed the adverse prognosis of myocardial enterovirus replication and the clinical importance of the disease (Why et al., 1994). Rapid diagnosis of myocardial enterovirus infection was achieved in recent years, since routine endomyocardial biopsy (Figulla et al., 1992) with subsequent enterovirus detection by reverse transcription and polymerase chain reaction (RT-PCR) was introduced as diagnostic procedure in suspected myocarditis and obscure sudden heart failure (Jin et al., 1990; Kämmerer et al., 1994). Therefore, a real chance of early intervention by antiviral therapy has emerged.

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazolo-3-carboxamide), is a broad-spectrum nucleoside analogue virustatic drug (Gilbert and Knight, 1986), which is effective in vivo against several diseases caused by RNA viruses, e.g. RSV bronchiolitis, measles, Lassa fever, and hepatitis C (Gilbert and Knight, 1986; Reichard et al., 1991; Koskinas et al., 1995). In vitro activity of ribavirin against enteroviruses was demonstrated early (Sidwell et al., 1979), but the precise mechanisms of ribavirin action against enterovirus has not yet been determined. In a mouse model of coxsackie virus myocarditis, antiviral effects of ribavirin were shown previously (Kishimoto et al., 1988). In contrast to the mouse model, in vitro evaluation of ribavirin against CVB3 in human cells (FL-amnion) resulted in a disappointingly low antiviral activity ($EC_{50} = 105.4$ μg/ml) (Okada et al., 1992) compared to the plasma concentrations that were obtained in pharmacokinetic studies (Lertora et al., 1991; Morse et al., 1993). However, the determination of a 2 $\log_{10}$ reduction of virus replication (EC_{99}) in organ specific cells may be a better objective for predicting potential clinical effects than EC_{50} values in permanent cell lines.

Propagation of non-transformed human myocardial fibroblasts from small myocardium samples of paediatric-surgical origin was described recently (Heim et al., 1995). Cultured human myocardial fibroblasts are a suitable and advantageous in vitro system for testing antiviral agents in enterovirus myocarditis for several reasons. First of all, infection of human myocardial fibroblast cultures with cardiotropic CVB3 results in persistent carrier-state virus replication with a high virus titer in the culture supernatant (Heim et al., 1995). In this type of infection, a small proportion of the cell population is productively infected, thereby resembling enterovirus infection of myocardial interstitial cells in vivo (Kandolf and Hofschneider, 1989, Koide et al., 1992). Second, propagation of myocardial fibroblasts of paediatric origin is easily obtained compared to propagation of fibroblasts from adults and results in a sufficient cell number for testing antiviral agents (Heim et al., 1995). In addition, enterovirus myocarditis is more prevalent and more severe in infants than in adults (Kaplan et al., 1983).

In contrast to fibroblasts, culture of cardial myocytes is currently not feasible in sufficient numbers for testing antiviral agents. Previous

studies with foetal cardial myoblasts demonstrated acute lysis of cells after infection with CVB3 and a protective effect of IFN-β (Kandolf et al., 1985a). Instead of IFN-β, which is also the most active interferon in human myocardial fibroblasts (Heim et al., 1996), IFN-α was selected for clinical studies (Figulla et al., 1995, Miric et al., 1995), because IFN-α has the advantage of yielding high plasma concentrations after simple subcutaneous administration (Wills, 1990). Previously, antiviral effects of IFN-α were demonstrated in the mouse model of enterovirus myocarditis (Matsumori et al., 1988) and in cultured human myocardial fibroblasts (Heim et al., 1995). As natural IFN-α has a higher antiviral activity than IFN-α2a (Heim et al., 1995), we also investigated whether a combination of ribavirin with natural IFN-α might be advantageous.

2. Material and methods

2.1. Experimental outline

Two lines of human myocardial fibroblasts (HJF and HXF) were used for determining the antiviral activity of ribavirin (HJF) and in a second experiment, the antiviral interaction of ribavirin with IFN-α (HXF). Cultures were infected with CVB3 1 week prior to application of antiviral agents. Different concentrations of ribavirin (25, 50 and 100 μg/ml) and IFN-α (10, 50, 250 and 1250 IU/ml) were examined separately or in combination over a period of 16 days, each in triplicate cultures. Culture media were changed every other day, and antivirals were added to fresh culture media. Supernatants at days 4, 8, 12, and 16 were frozen to -20°C and used for virus titration by plaque assays. On day 16, cells were harvested by incubation with versene for quantitative in situ hybridization and determination of intracellular CVB3 RNA concentration by slot blot hybridization.

2.2. Cells, virus and antiviral agents

Human myocardial fibroblasts (HJF and HXF) were prepared from paediatric-surgical myocardial tissue of two individuals without enzymatic tissue disaggregation as described recently (Heim et al., 1995). Fibroblasts were propagated with Dulbecco's modified Eagle's minimal medium (DMEM), supplemented with 10% foetal bovine serum (FBS; Life technologies, Grand Island, NY), non-essential amino-acids, 4.5 g glucose/l and 50 μg kanamycin/ml. Fibroblast cultures were controlled for absence of mycoplasma contamination with a nucleic acid hybridization kit (Geneprobe, San Diego, CA). Both myocardial fibroblast lines stained uniformly positive for the fibroblast marker enzyme prolyl-4-hydroxylase (monoclonal antibody (MAB) 5B5, Dako, Glostrup, Denmark), but negative for an endothelial cell marker (MAB CD31, JC/70A, Dako) after visualization with a standard APAAP (alkaline phosphatase/anti phosphatase) technique (Dako). Controls using normal serum were done to exclude non-specific staining. Coxsackie virus B3 (CVB3) was derived by transfection from the plasmid CVB3-M1 (Kandolf and Hofschneider, 1985b) which contains the complete genome of CVB3 (cardiotropic Nancy strain). Virus was plaque-purified on Vero cells (ATCC No.CCL 81) as described previously (Kandolf et al., 1985a). Confluent monolayers of human myocardial fibroblasts were infected with plaque-purified CVB3 at a multiplicity of infection (MOI) of 0.5 pfu/cell. Ribavirin was provided by ICN Europe (Bucks, UK). Human, highly purified, natural interferon-α with a specific activity of 1.4×10^8 IU/mg was provided by Rentschler (Laupheim, FRG).

2.3. Virus yield assays

Infectious virus titers in culture supernatants of persistently CVB3-infected human myocardial fibroblasts were quantitatively determined by plaque assays as described previously (Kandolf et al., 1985a). Vero cells were used for plaque assays, as they were insensitive to a carry-over of up to 500 μg ribavirin/ml and 6250 IU IFN-α/ml.

2.4. Sensitivity assays

Ribavirin-sensitivity of coxsackie B3 virus stock and CVB3 derived from fibroblast cultures treated

for 16 days with ribavirin was assayed by a plaque reduction method as 50% inhibitory concentration (IC_{50}). Briefly, confluent monolayers of HeLa S3 cells (ATCC CCL 2.2) were treated with various concentrations of ribavirin for 4 h, washed with PBS, infected with 100 pfu CVB3 and overlaid with 1% seaplaque agarose (FMC) in DMEM (supplemented with 5% FBS, 20 mM $MgCl_2$, and 50 μg kanamycin/ml) containing various concentrations of ribavirin. Cells were fixed after 48 h with 5% trichloroacetic acid, stained with 1% crystal violet and examined for plaque count.

2.5. Slot-blot hybridization and in situ hybridization

At 16 days after addition of the antiviral agents, fibroblasts were harvested with versene. Total RNA was prepared from 1×10^6 cells as described by Chirgwin et al., 1979; 250 ng RNA was applied to nitrocellulose with a slot-blot device. A DNA probe consisting of about 90% of the virus genome (0.06–7.2 kB) was ^{32}P-labeled by nick-translation (Kandolf and Hofschneider, 1985b). Slot blot hybridization was performed according to standard procedures (Sambrook et al., 1989) and RNA extracted from non-infected fibroblasts was used as negative control, RNA from infected, untreated cultures as positive control. Hybridization signals were quantified using a LKB/Pharmacia (Sollentuna, Sweden) laser densitometer with a serial dilution of the positive control as standard. For in situ hybridization, 1×10^5 cells were applied each to microscopic slides. In situ hybridization was performed as described recently (Heim et al., 1992) using the DNA probe described above labeled with [^{35}S] by nick-translation. Slides were examined unstained with an interference-contrast microscope (Zeiss Jenaval, Jena, FRG). A hundred cells were counted on each slide at least three times. Counting was performed in a blinded fashion to avoid observer bias. Degree of infection was determined as percent of positive cells according to the ratio: (positive cells/(negative cells + positive cells)) × 100%. Intraobserver error and interobserver error were obtained from repeated measurements and expressed as the percentage of total variance between slides. Estimated percentages of variance were low in both cases (5.3 and 5.7%, respectively) and confirm the reliability of the employed method.

2.6. Mathematical evaluation of drug interactions

The multiple drug effect analysis of Chou and Talalay (1984) was used to evaluate combined drug effects. Median effect plots (regression lines) of each set of ribavirin and IFN-α dose-effect data were calculated. The significance of the correlation coefficient of each regression line was controlled (Fisher, 1973). The slope (m) of the median-effect plot, which signifies the shape of the dose-effect curve and the dose of the median effect (D_m) was then used for the calculation of concentrations resulting in a 90 or 99% reduction of virus yields (EC_{90} or EC_{99}, respectively) and for calculation of combination index values (CI) of the ribavirin/interferon-α combinations (Chou and Talalay, 1984). Standard deviations (S.D.) of the EC_{90}, EC_{99} and of the CI were calculated using the Monte-Carlo principle as described recently (Belen'kii and Schinazi, 1994, Heim et al., 1996). The condition $CI - (1.96 \times S.D.) > 1$ indicates antagonism, $CI + (1.96 \times S.D.) < 1$ indicates synergism and additivity is indicated by $CI - (1.96 \times S.D.) < 1$ or $CI + (1.96 \times S.D.) > 1$.

2.7. Cell-proliferation and viability assays

Reduction of cell proliferation by ribavirin or IFN-α was quantitatively evaluated with the help of the cleavage of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium-bromide (MTT) to dark blue formazan by viable cells (Denizot and Lang, 1986). Briefly, human myocardial fibroblasts were incubated for a period of 4 days with various concentrations of IFN-α (10–8000 IU/ml) and ribavirin (25–3200 μg/ml) either as single agents or in combination, each in quadruplicates. Media were removed and cells were incubated with 50 μg/ml MTT in phenol red and FBS-free DMEM for 4 h. Untransformed MTT-DMEM was removed and formazan was extracted from the cell-monolayer by incubation with isopropanol. Absorbance was quantified at a wavelength of 560

nm in a standard photometer (reference wave length 690 nm).

3. Results

3.1. Antiviral effects of ribavirin

Ribavirin sensitivity (IC_{50}) of transfection-derived CVB3 (Nancy strain) used for infection of human myocardial fibroblasts was 77 μg/ml as determined with HeLa cells. In contrast to this result, ribavirin was highly active against CVB3 carrier replication in human myocardial fibroblasts (HJF, Fig. 1). Antiviral effects of ribavirin in CVB3 carrier cultures of human myocardial fibroblasts were significantly concentration-dependent ($P < 0.01$, correlation coefficient of regression line). The high antiviral activity of ribavirin was confirmed with another line of human myocardial fibroblasts (HXF, Fig. 2A), which had higher virus titers after carrier state infection with CVB3 (2.1×10^6 pfu/ml, S.D. $= 1.3 \times 10^6$; vs. 1.7×10^4 pfu/ml, S.D. $= 1.2 \times 10^4$ in the HJF-line). For simplified comparison of the ribavirin effects, EC_{99} values were calculated from the data presented in Figs. 1 and 2(a). This resulted in almost identical antiviral affectivity in both lines of myocardial fibroblasts (Table 1). Moreover, a complete suppression of infectious virus replication was achieved in both fibroblast cell lines with 100 μg ribavirin/ml on day 16 in two of the three

Fig. 1. Reduction of virus titers in CVB3 carrier cultures of human myocardial fibroblasts (HJF cells; mean values of triplicate cultures, error bars indicate S.D.). Infected control (open bar), 25 μg ribavirin/ml (hatched bar), 50 μg ribavirin/ml (dotted bar), 100 μg ribavirin/ml (solid bar). * Complete suppression of infectious virus replication in two of three cultures, mean virus titer less than 1 pfu/ml.

Fig. 2. Reduction of virus titers in CVB3 carrier cultures of human myocardial fibroblasts over a period of 16 days by ribavirin (A), by a combination of ribavirin and IFN-α (B), and IFN-α (C) as a single agent (HXF-cells; mean values of triplicate cultures, error bars indicate standard deviation). A: infected control (open bar); 25 μg ribavirin/ml (hatched bar); 50 μg ribavirin/ml (dotted bar); 100 μg ribavirin/ml (solid bar). B: infected control (open bar); 25 μg ribavirin plus 10 IU IFN-α/ml (hatched bar); 25 μg ribavirin plus 50 IU IFN-α/ml (dotted bar). C: infected control (open bar); 10 IU/ml (hatched bar); 50 IU/ml (criss-cross bar); 250 IU/ml (dotted bar); 1250 IU/ml (solid bar). * Complete suppression of infectious virus replication in two of three cultures.

Table 1
Ribavirin concentrations (μg/ml) resulting in a 2 $\log_{10}$ reduction of virus yields (EC_{99}) in CVB3 carrier-cultures of human myocardial fibroblasts after different periods of ribavirin application

Line of fibroblasts	EC_{99} on			
	Day 4	Day 8	Day 12	Day 16
HJF	54 (S.D. = 5.6)	36 (S.D. = 1.3)	40 (S.D. = 5.8)	49 (S.D. = 5.6)
HXF	62 (S.D. = 14)	41 (S.D. = 4.0)	48 (S.D. = 3.1)	42 (S.D. = 2.0)

EC_{99} calculated from data presented in Fig. 1 (HJF cells), and from data presented in Fig. 2A (HXF cells).

cultures. In contrast to the results obtained with HJF cells (Fig. 1), an increase of virus titers was noted in HXF cells treated with 25 or 50 μg ribavirin/ml after 16 days (Fig. 2A). However, ribavirin-sensitivity assays with CVB3 derived of HXF cells on day 16 demonstrated an almost equal sensitivity ($IC_{50} = 71$ μg/ml) compared to the stock virus ($IC_{50} = 77$ μg/ml).

In addition to virus yields, the effect of ribavirin on intracellular CVB3-RNA concentration was quantitatively assessed. Slot-blot hybridization demonstrated a concentration-dependent reduction of CVB3 RNA in human myocardial fibroblasts after 16 days of ribavirin application (Fig. 3). Laser-densitometry of the blots displayed a 68% reduction of intracellular CVB3 RNA by 50 μg ribavirin/ml and a greater than 87.5% reduction by 100 μg/ml compared to infected controls, whereas with 25 μg/ml a significant effect on CVB3 RNA was not observed (Fig. 3). In contrast to the results on CVB3 RNA concentration, the number of infected cells was reduced slightly by 25 μg ribavirin/ml over a period of 16 days as determined by in situ hybridization (Table 2). The effects of ribavirin on the number of infected cells in CVB3 carrier cultures were significantly concentration-dependent ($P < 0.1$, correlation coefficient of regression line) resulting in a maximum reduction to 0.3% positive cells with 100 μg ribavirin/ml compared to 14.3% in control cultures.

Fig. 3. Slot blot hybridization: A, 25μg ribavirin/ml; B, 50 μg ribavirin/ml; C, 100 μg ribavirin/ml; D, infected control; E, negative control; F, infected control (1:2 diluted); G (1:4 diluted); H (1:8 diluted).

3.2. Antiviral effects of ribavirin combined with IFN-α

The reduction of virus yields by the combination of 25 μg ribavirin/ml with either 10 or 50 IU IFN-α/ml is depicted in Fig. 2B. For comparison, the reduction of virus yield by ribavirin and IFN-α as single agents are shown in Fig. 2A and C, respectively. Results of drug interaction analysis indicated antiviral additivity of ribavirin with IFN-α in reducing virus yields on any day tested, with exception of day 8 after starting application of ribavirin and IFN-α (Table 3). On day 8, ribavirin and IFN-α were slightly synergistic in reducing virus yields (Table 3). In addition to the reductions of virus titer, the combined effects of ribavirin and IFN-α on the number of infected cells in carrier cultures were studied by quantitative in situ hybridization. For example, the combination of 25 μg ribavirin plus 50 IU IFN-α/ml decreased the percentage of infected cells to 2.6%, compared to 8.9% with 25 μg ribavirin/ml or 6.2% with 10 IU IFN-α/ml as single agents and to 14.3% in the infected controls (Table 2). Hence, calculation of CI-values resulted in a slightly synergistic interaction (CI = 0.82, S.D. = 0.03) with the number of infected cells.

Table 2
Percentage of CVB3 RNA-positive cells in CVB3 carrier cultures as determined by quantitative in situ hybridization 16 days after starting application of ribavirin and IFN-α

Ribavirin (μg/ml)	IFN-α (IU/ml)	Infected cells[a] (%)	Drug interaction[b] (CI)
0	0	14.3 (S.D. = 1.7)	
25	0	8.9 (S.D. = 1.5)	
50	0	5.4 (S.D. = 2.5)	
100	0	0.3 (S.D. = 0.2)	
0	10	6.2 (S.D. = 2.4)	
0	50	5.2 (S.D. = 2.6)	
0	250	3.7 (S.D. = 0.5)	
0	1250	0.4 (S.D. = 0.2)	
25	10	5.6 (S.D. = 0.3)	1.00 (S.D. = 0.04)
25	50	2.6 (S.D. = 1.1)	0.82 (S.D. = 0.03)

[a] Mean of at least three slides.
[b] Drug interaction calculated as proposed by Belen'kii and Schinazi (1994).

Moreover, the combination of 25 μg ribavirin/ml with either 10 IU IFN-α/ml (or 50 IU IFN-α/ml) resulted in a significant 65% (or 71%, respectively) reduction of cellular CVB3 RNA as determined by slot-blot hybridization, whereas no significant reduction of CVB3 RNA was observed with either 25 μg ribavirin or up to 50 IU IFN-α/ml as single agents (Fig. 3). However, mathematical analysis of the drug-interaction in reducing CVB3 RNA concentration was not feasible due to the lack of significant concentration-dependent effects of IFN-α on CVB3 RNA concentration.

3.3. Anti-proliferative effects of ribavirin

Anti-proliferative effects of both ribavirin and IFN-α were low (less than 50% with concentrations as high as 3200 μg ribavirin/ml and 80 000 IU IFN-α/ml) and not enhanced if both agents were applied in combination. This confirms that antiviral effects of both agents are highly specific and not related to cytotoxicity.

4. Discussion

Effective antiviral therapy of myocardial enterovirus infections has not yet been established, although this seems to be urgently required in view of the adverse prognosis of the disease (Why et al., 1994). In the present study, the antiviral activity of ribavirin was demonstrated for the first time in a species- and organ-specific in vitro model of persisting CVB3 replication. EC_{99} values were as low as 36 μg/ml and EC_{90} values even less than 25 μg/ml. Our results are in agreement with a previous study that demonstrated a high antiviral activity of ribavirin in a persistent in vitro infection with an animal enterovirus, foot- and mouth-disease virus (FMDV) (de la Torre et al., 1987). Similar to the results obtained with FMDV, CVB3 carrier cultures may even be 'cured' from infectious virus progeny by concentrations of at least 100 μg ribavirin/ml (Figs. 1 and 2A). In contrast to our results, the recently reported antiviral activity of ribavirin against CVB3 in other human cells (FL amnion cells) was low (EC_{50}, 105.4 μg/ml), and it was even impossible to achieve a 2 $\log_{10}$ reduction of virus yields (EC_{99}) with concentrations ten-fold higher (400μg/ml) as EC_{99} in myocardial fibroblasts (Okada et al., 1992). There are two possible explanations for the divergent results on ribavirin activity: (i) ribavirin has a cell-type specific high activity in human myocardial fibroblasts; or (ii) a highly ribavirin-susceptible strain of CVB3 was used in the present study. Since both the present and the previous study used the cardiotropic Nancy strain of CVB3, cell specific properties of myocardial fibroblasts are obviously the explanation for the higher activity of ribavirin in our study. This hypothesis is confirmed by the rib-

Table 3
Combination index values for the reduction of virus yields of the combination of 25 μg ribavirin/ml with either 10 (CI_L) or 50 IU IFN-α/ml (CI_H), determined after various periods of application

Day	4	8	12	16
CI_L (S.D.)	0.96 (0.13)	0.85 (0.04)	1.21 (0.62)	0.92 (0.18)
CI_H (S.D.)	1.12 (0.13)	0.91 (0.04)	1.08 (0.24)	0.79 (0.12)

S.D., standard deviation of CI.

avirin sensitivity assays using HeLa S3 cells, which resulted in a comparatively low ribavirin susceptibility of our virus stock ($IC_{50} = 77$ μg/ml).

Although the mode of antiviral action of ribavirin is still poorly understood, riba-virin may act as a nucleoside triphosphate analogue inhibitor of the RNA-dependent enteroviral polymerase besides other potential antiviral mechanisms (Patterson and Fernandez-Larsson, 1990). Irrespective of the precise mechanism, the antiviral action in a definite cell line is dependent on a high intracellular concentration of ribavirin-phosphates compared to the nucleoside triphosphate (especially guanosine triphosphate) concentration. The cellular ribavirin triphosphate concentration is influenced by cell-specific rates of uptake and phosphorylation in relation to the hydrolysis back to ribavirin (Gilbert and Knight, 1986). Therefore, antiviral activity of ribavirin is cell-specific (Patterson and Fernandez-Larsson, 1990), for example, antiviral activity was high in human myocardial fibroblasts, low in HeLa cells and absent in Vero cells (fibroblastoid monkey kidney cells, data not shown) in the present study. Cell-type specific ribavirin activity is also important in vivo, as ribavirin inhibits hepatitis C virus replication sufficiently in hepatic cells, but not in peripheral mononuclear leukocytes (Koskinas et al., 1995). In contrast to the high antiviral activity of ribavirin, human myocardial fibroblasts were almost insensitive to anti-proliferative effects of ribavirin ($< 50\%$ growth inhibition at ribavirin concentrations as high as 3200 μg/ml). Probably, this finding is due to the slow proliferation of myocardial fibroblasts in culture compared to transformed cell lines (e.g. HeLa), since the cytotoxic activities of the nucleic acid analogue drugs are more pronounced in rapidly proliferating cells.

Besides cell-type specific properties of myocardial fibroblasts, the multiple infection cycles in carrier state cultures may be very susceptible to virustatic activities of antiviral agents in contrast to the acute type of infection in FL or HeLa cells. Multiple rounds of CVB3 replication in carrier-cultures and continuous application of ribavirin over a period of 16 days seem to be favourable prerequisites for rapid in vitro selection of drug resistant virus strains. Indeed, in one line of myocardial fibroblasts (HXF), a decrease of the antiviral effects of 25 and 50 μg ribavirin/ml was observed on day 16 (Fig. 2A). However, this was not caused by a reduced ribavirin sensitivity of CVB3. Therefore, reduced antiviral effects of ribavirin may be explained by a decreased intracellular ribavirin-phosphates/GTP ratio after more than 12 days of ribavirin application. This indicates the possibility of a cellular 'ribavirin resistance' associated with prolonged ribavirin presence in some cells.

In contrast to plaque assays, sensitive nucleic acid hybridization techniques demonstrated persistence of CVB3 RNA even in the 100 μg ribavirin/ml schedule (Table 2), which may result in reactivation of the infection after cessation of ribavirin application. For example, reactivation of infectious CVB3 replication by persisting CVB3 RNA was observed in a similar cell culture system (myocardial fibroblasts of foetal origin) after cessation of interferon-application previously (Heim et al., 1992). Therefore, nucleic acid hybridization techniques seem to be a useful tool for determining long term effects of antiviral agents in persistently infected cell cultures. Especially in situ hybridization may be proposed for future studies, as both minor effects of low ribavirin concentrations (25 μg/ml) and persistence of a few infected

cells with 100 μg ribavirin/ml were quantitatively detected (Table 2). Low standard deviations of quantitative in situ hybridization data enabled calculation of CI values for determining interaction of antivirals (Chou and Talalay, 1984; Belen'kii and Schinazi, 1994). This resulted in a slightly synergistic interaction of 25 μg ribavirin with 50 IU IFN-α/ml and an additive interaction with 10 IU IFN-α/ml. Although slot blot hybridization demonstrated a significant reduction of CVB3 RNA with combination schedules compared to single agents, use of slot blot hybridization for determining antiviral effects has several drawbacks compared to in situ hybridization. For example, antiviral effects of the low dose (25 μg ribavirin/ml) schedule were not detected by slot-blot hybridization. Moreover, it was impossible to determine a concentration-dependent effect of IFN-α on CVB RNA concentration. This may be in part due to the more pronounced effects of interferon on protein synthesis and infectious virus progeny than on RNA replication (Baron and Dianzani, 1994).

Since a synergistic interaction of ribavirin with recombinant IFN-α A/D was reported recently (Okada et al., 1992), we investigated whether the additive (at best slightly synergistic) interaction in reducing infectious virus progeny in myocardial fibroblasts was a consequence of the use of natural IFN-α instead of recombinant IFN-α. IFN α-2a was selected as recombinant subtype of IFN-α instead of IFN-α A/D, because the antiviral activity of IFN α-2a was already demonstrated in human myocardial fibroblasts (Heim et al., 1995). Furthermore, IFN α-2a was used in a clinical study on enteroviral heart disease recently (Figulla et al., 1995). Similar to the results obtained with natural IFN-α, an additive interaction of 50 IU IFN-α-2a with 25 μg ribavirin/ml was observed after 4 and 8 days of application (CI = 0.94, S.D. = 0.05). Thus, it can be concluded that both recombinant and natural IFN-α interact additively with ribavirin in human myocardial fibroblasts. Nevertheless, an additive combination of antiviral agents may be a suitable strategy, if dose-dependent side-effects of both agents are different (as e.g. with ribavirin and IFN-α) and consequently dose-limiting side-effects can be avoided (Bocci, 1994; Morris, 1994). For example, the combination of ribavirin with IFN-α in hepatitis C results in a significantly higher antiviral and clinical efficacy than therapy with IFN-α or ribavirin as single agents (Brillanti et al., 1994; Schvarcz et al., 1995).

Although EC_{99} values of ribavirin determined in myocardial cells were ten-fold lower than reported previously in FL amnion cells (Okada et al., 1992), ribavirin plasma concentrations achieved with oral high dose schedules are still about five to ten-fold lower (Lertora et al., 1991; Morse et al., 1993). However, sufficient ribavirin concentrations may be achieved in vivo with intravenous application (Morris, 1994).

In conclusion, our results suggest that the use of heart cells and conditions of persistent infection may be advantageous in testing ribavirin and other antiviral agents in future studies on enterovirus myocarditis.

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