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REGULATION OF INTERFERON PRODUCTION. SUPERINDUCTION BY DIHYDRORIFAMPICIN IN HUMAN AND CHICK EMBRYO FIBROBLASTS AND MOUSE L929 CELLS

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Summary. — Dihydrorifampicin (DHR), a new reversible inhibitor of RNA polymerases I and II in eukaryotic cells, exhibited a very high enhancing effect on the production of interferon (IFN) in cultures of human and chick embryo fibroblasts and mouse L929 cells induced by poly(I). poly(C). The titres of interferon produced in human fibroblast cultures superinduced with poly(I). poly(C), cycloheximide and DHR were 128 times higher as compared with cultures treated with poly(I). poly(C) only. A similar superinduction of interferon by DHR was observed in mouse and chicken cell cultures, IFN titres in culture media were 40—60 times higher in comparison with cultures treated only with the inducer. In comparison with actinomycin D in the superinduction experiments, DHR was not toxic and allowed much higher yields of IFN. The use of DHR may be especially advantageous for the superinduction and production of human fibroblast IFN in cultures of human diploid fibroblasts.

Key words: *interferon*; *superinduction*; *dihydrorifampicin*

Introduction

IFN production in cultures of diploid human fibroblasts induced by the synthetic double-stranded RNA, polyriboinosinic-polyribocytidylic acid poly(I). poly(C), is markedly enhanced by appropriate exposure to inhibitors of RNA or protein synthesis, a phenomenon called "superinduction" (Vilček *et al.*, 1976; Novokhatsky *et al.*, 1977; Sehgal and Tamm, 1978; Tamm and Sehgal, 1978). Actinomycin D and cycloheximide increased IFN production when added during or before the shut off phase of IFN production to cell cultures treated with poly(I). poly(C) (Myers and Friedman, 1971; Tan *et al.*, 1971; Havell and Vilček, 1972; Billiau *et al.*, 1973; Vilček and Havell, 1973; Vilček *et al.*, 1976). The half-life of IFN mRNA in the cells is concomitantly increased by superinducing treatments (Vilček and Havell, 1973; Cavalieri *et al.*, 1977; Sehgal *et al.*, 1978). The increased functional stability

of IFN mRNA possibly results from an inhibition by RNA synthesis inhibitors of the synthesis of mRNA of a rapidly turning-over repressor involved in the inactivation or degradation of IFN mRNA (Vilček *et al.*, 1976; Sehgal *et al.*, 1978). The superinducing treatments prolong the period of accumulation of intracellular IFN mRNA and decrease the rate of degradation of this mRNA (Cavalieri *et al.*, 1977). The enhancing effect of 5, 6--dichloro-l-β-D-ribofuranosylbenzimidazole (DRB), a selective and reversible inhibitor of nuclear heterogeneous RNA and messenger RNA synthesis on IFN production by poly (I).poly(C)-induced human fibroblasts has also been described (Sehgal *et al.*, 1975; Sehgal *et al.*, 1976*a*,*b*: Sehgal *et al.*, 1977, 1978; Tamm and Sehgal, 1978).

In this communication we describe the enhancing effect of dihydrorifampicin (DHR), a reversible inhibitor of RNA polymerases I and II of eukaryotic cells (Kára and Černá, 1976; Kára and Hostomský, 1979) on the production of IFN in cultures of human and chick embryo fibroblasts and mouse L929 cells induced by poly(I). poly(C).

DHR, a non-toxic derivative of the antibiotic rifampicin, has been synthesized by catalytic hydrogenation of the 18, 19 carbon atoms in the aliphatic ansa chain of the rifampicin molecule (Hanuš *et al.*, 1979) and has been shown to inhibit selectively RNA synthesis, but not DNA synthesis in HEp-2 human tumor cells and chick embryo fibroblasts (Kára and Černá, 1976).

DHR inhibits messenger RNA and ribosomal RNA synthesis but not tRNA synthesis in HEp-2 cells (Kára and Hostomský, 1979) and its cytostatic effect is fully reversible (Kára and Černá, 1976). These properties of DHR seem to be advantageous, in comparison with actinomycin D, in experiments on the superinduction of IFN, because DHR is not toxic and the cells treated with DHR survive. The superinduction of IFN by DHR in human diploid fibroblast cultures may therefore be repeated in order to increase the yield of IFN.

Materials and Methods

Cell cultures. Cultures of human embryo fibroblasts (HEF), chick embryo fibroblasts (CEF) and mouse L929 cells were provided by the Laboratory of Tissue Cultures of the D. I. Ivanovsky Institute of Virology, Academy of Medical Sciences U.S.S.R. Cells were grown in vials as described previously (Ershov *et al.*, 1971). Human and mouse cells were maintained by weekly passages in medium 199 containing 10% foetal calf or bovine serum in the presence of antibiotics.

Chemicals. The polyinosinic. polycytidylic acid preparation was purchased from Calbiochem (U.S.A.) and DEAE-dextran (mol. wt. 5×10^5) from Pharmacia (Uppsala, Sweden). Dihydrorifampicin (DHR) was synthesized in the Institute of Nuclear Biology, Czechoslovak Academy of Sciences, Prague, and kindly provided by the authors (Hanuš *et al.*, 1979). A fresh stock solution of DHR in dimethyl sulphoxide (5—10 mg per ml) was prepared before each experiment and further dilutions were made in tissue culture medium 199. Actinomycin D and cycloheximide (Calbiochem, U.S.A.) were dissolved in sterile distilled water and diluted in medium 199 to the concentrations indicated below.

IFN induction. Cell cultures were exposed for 1 hr to poly(I). poly(C) (50 μg per ml) in medium 199 containing 50 μg per ml of DEAE-dextran at 37 °C. The cultures were then washed three times with medium 199 to remove the inducer and replenished with 1 ml of medium 199 containing 2% bovine serum. Culture fluids were collected after 24 hr of incubation at 37 °C and pooled from 3—5 vials. Interferon was assayed in these pooled samples.

Table 1. Superinduction of IFN production by DHR and other inhibitors of RNA and protein synthesis in HEF, CEF and L929 cells

Conditions of the induction and superinduction of IFN in the cell cultures. Time of treatment of cells with the inducer and inhibitors (in hr)				Titre of IFN in media of treated cultures (units/ml)		
poly(I).poly(C) (50 μg/ml)	cycloheximide (20 μg/ml)	actinomycin D (2 μg/ml)	DHR (50 μg/ml)	HEF	L929	CEF
0—1	—	—	—	10	80	40
0—1	1—5.5	5.5—6	—	80	320	640
0—1	1—5.5	—	5.5— 7.5	1,280	2,560	2,560
0—1	1—5.5	—	5.5—11.5	40	160	320

For details on IFN induction and superinduction see section "Materials and Methods".

Superinduction of IFN. Confluent cell monolayers were treated at 37 °C with poly(I). poly(C) (50 μg per ml) for 1 hr in medium 199 containing DEAE-dextran (50 μg per ml). The cultures were then washed with medium 199 to remove the inducer and further incubated for 4 hr and 30 min in medium containing cycloheximide (20 μg per ml). After removal of cycloheximide, the cultures were incubated in medium containing either actinomycin D (2 μg per ml) for 30 min, or DHR (50 μg or 100 μg per ml) for 1/2 to 24 hr. The 2 hr period of treatment of cells with DHR was found to be optimal, longer incubation of cultures in medium with DHR resulted in a decrease IFN production. The inhibitors were removed from the cultures by washing the cell monolayers three times with 2 ml of medium 199 and cultures were incubated for further 24 hr at 37 °C in fresh medium 199 containing 2% bovine serum. Culture fluids were then collected and pooled from 3 vials and assayed for IFN titre.

IFN assay. Human and mouse IFN were assayed on homologous cells (human embryonic lung cells and mouse L929 cells) using vesicular stomatitis virus (VSV) strain Indiana and Venezuelan equine encephalomyelitis virus (VEEV) by described methods reducing the cytophatic effects (Sadykov *et al.*, 1978). Chick IFN was assayed by a plaque reduction method using VEEV in cultures of chick embryo fibroblasts (Sadykov *et al.*, 1978).

One unit of IFN was defined as the reciprocal of the highest dilution of a sample that inhibits the cytopathic effect of the viruses in 50% of cells or reduce VEEV plaque fromation in CEF cultures by 50%. IFN titres were expressed in units per ml.

Results

We compared the effects of actinomycin D and DHR in combination with poly(I).(poly(C) and cycloheximide on IFN superinduction in cultures of HEF, mouse L929 cells and CEF. Cultures were treated with poly(I). (poly(C) and inhibitors as described in "Materials and Methods". We found a very high IFN-superinducing effect of DHR, i.e., a high increase of IFN production after treatment of induced cultures with cycloheximide and DHR (Table 1). Treatment of induced HEF cultures with actinomycin D or DHR

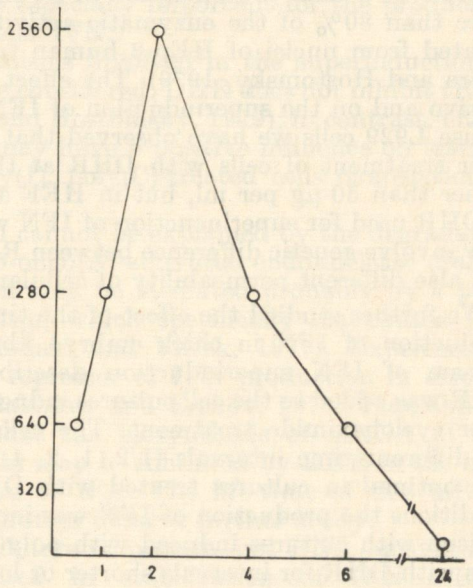

Fig. 1.

Effect of the incubation time with DHR on IFN production in CEF cultures The cells were induced with poly(I). .poly(C) (100 μg/ml) for 1 hr and then treated with cycloheximide (20 μg/ml) for 4 hr and 30 min. The cells were then incubated in medium containing DHR (50 μg/ml) for 1/2, 1, 2, 4, 6, and 24 hr. Culture fluids were collected 24 hr after removing the inhibitor and the IFN titres were assayed. The titre of IFN in medium of CEF cultures induced with poly(I).poly(C) was 40—80 units/ml at 24 hr after induction.
Abscissa: hr of treatment of cells with DHR; ordinate: IFN units per ml.

resulted in stimulation of IFN production 8 times and 128 times, respectively (Table 1). The superinducing effect of DHR on the production of human fibroblast was therefore much higher in comparison with actinomycin D. This effect may have been due to interference of DHR with the molecular mechanism of the shutoff phenomenon, probably by inhibition of mRNA biosynthesis of a hypothetical repressor and stabilization of IFN mRNA (Vilček and Havell, 1973; Vilček *et al.*, 1976; Cavalieri *et al.*, 1977; Sehgal *et al.*, 1978).

Whilst actinomycin D, a highly toxic antibiotic, binds to cellular DNA and inhibits irreversibly the cellular RNA biosynthesis (Reich and Goldberg, 1964), DHR is a non-toxic inhibitor of eukaryotic RNA polymerases I and II (Kára and Hostomský, 1979), inhibiting reversibly the intracellular RNA biosynthesis only during the short period of the contact of DHR with the cells. The cells treated with DHR maintained their full metabolic and biosynthetic capacity. This may explain the higher efficiency of DHR in the superinduction of IFN production, in comparison with actinomycin D.

IFN levels in cultures of mouse L929 and chick embryo cells superinduced by DHR and cyclohexamide were reproducibly 40—60 times higher as compared with cultures treated with poly(I). poly(C) only (Table 1). The phenomenon of IFN superinduction using actinomycin D and cycloheximide in mouse and chicken cells have not been so regularly observed (Novokhatskij *et al.*, 1977). Treatment of induced cells with DHR alone resulted also in stimulation of IFN production but IFN levels were lower in comparison with cultures treated with poly(I). poly(C), cycloheximide and DHR (data not shown).

DHR at a final concentration of 80 μg per 0.25 ml was found to inhibit more than 80% of the enzymatic activities of RNA polymerases I and II isolated from nuclei of HEp-2 human tumor cells and Rous sarcoma cells (Kára and Hostomský, 1979). The effect of the drug on the RNA synthesis in vivo and on the superinduction of IFN was dependent on a cell type. In mouse L929 cells we have observed that the production of IFN was higher after treatment of cells with DHR at the concentration of 100 μg per ml rather than 50 μg per ml, but in HEF and CEF the optimal concentration of DHR used for superincuction of IFN was 50 μg per ml. This phenomenon may involve genetic difference between RNA polymerases of different species and also different permeability of cellular membranes for DHR.

We further studied the effect of the time of incubation with DHR on the production of IFN in chick embryo fibroblast cultures. According to the scheme of IFN superinduction described in "Materials and Methods", DHR was added to the cell cultures induced by poly(I). (poly(C) immediately after cycloheximide treatment. The cells were then incubated with DHR for different time intervals (1/2, 1, 2, 4, 6, and 24 hr). Production of IFN was optimal in cultures treated with DHR for 2 hr (Fig. 1). Under these conditions the production of IFN was increased more than 30 times in comparison with cultures induced with poly(I). poly(C) only. The treatment of cells with DHR for intervals shorter or longer than 2 hr resulted in a decline

of IFN production (Fig. 1). This time-dependence of the treatment of cells with DHR and of superinduction of IFN probably involves inhibition by DHR of the biosynthesis of a rapidly turning-over messenger RNA of a repressor and indicates the requirement of a normal metabolic state of the cells for IFN biosynthesis after temporary and reversible inhibition of cellular RNA biosynthesis by DHR.

Discussion

The data presented in this communication show clearly that DHR may be used for superinduction of IFN in different eukaryotic cells with better efficiency than actinomycin D.

The use of DHR for superinduction of IFN offers several advantages. The titres of IFN produced in cells superinduced with poly(I). poly(C), cycloheximide and DHR are several times higher in comparison with conventional method using poly(I). poly(C), cycloheximide and actinomycin D (Havell and Vilček, 1972; Vilček *et al.*, 1976). This is very important for the production of human fibroblast IFN in cultures of human diploid cells, where superinduction by DHR resulted in an 100 fold higher yield of IFN (Table 1).

In comparison with actinomycin D, a highly toxic antibiotic, DHR is a non-toxic, semisynthetic antibiotic inhibiting reversibly RNA polymerases I and II in eukaryotic cells (Kára and Hostomský, 1979), and cells treated for 24 hr with DHR survive (Kára and Černá, 1976). We presume, therefore, that DHR may be repeatedly used in the same culture for superinduction of IFN, as was demonstrated with 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (Wiranowska-Stewart *et al.*, 1977). This possibility has to be proved experimentally; it would be especially important for the production and higher yields of human fibroblast IFN.

Concerning the molecular mechanisms involved in the superinduction of IFN by DHR, two aspects should be considered. DHR does not inhibit tRNA synthesis in the treated cells (Kára and Hostomský, 1979); in contrast, tRNA synthesis is rather increased. This may have a positive influence on translation of mRNAs present in the poly(I). poly(C)-treated cells (translation of IFN mRNA).

Because the shut off phenomenon cannot be explained by the degradation of IFN mRNA mediated by the 2,5-oligo(A)-activated endonuclease (Sehgal and Gupta, 1980), this phenomenon may be mediated probably by a post-transcriptional regulatory mechanism, which specifically inactivates IFN mRNA by a specific repressor (Kohase and Vilček, 1977). Experimental evidence for the existence of such repressor of IFN production in chicken cells was published recently (Tazulakhova and Ershov, 1978; Tazulakhova *et al.*, 1980*a*), It seems possible that the biosynthesis of mRNA of this repressor (Tazulakhova *et al.*, 1980b) may be inhibited by DHR in the molecular mechanism of superinduction of IFN. The life-time of this mRNA and the turnover of the repressor protein deserve further study.

Conventional methods using inhibitors of protein and RNA biosynthesis for superinduction of IFN resulted in stabilization of IFN mRNA

(Vilček and Havell, 1973; Cavalieri *et al.*, 1977; Sehgal *et al.*, 1978). A similar stabilization of IFN mRNA may be presumed in cells superinduced by DHR. The method using DHR for superinduction of IFN as described in present communication may facilitate the isolation of human fibroblast IFN mRNA from superinduced human diploid fibroblasts. This possibility has to be proved in further experiments.

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