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INTERFERON INDUCED INHIBITION OF QUEUINE UPTAKE IN CULTURED HUMAN FIBROBLASTS

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Interferon inhibits uptake of the radiolabeled queuine analog, rQT_3, into cultured human fibroblasts. Simultaneous exposure to 10 nM phorbol-12,13-didecanoate (PDD) potentiates interferon-induced inhibition of rQT_3 into cultured fibroblasts. All three major classes of human interferon tested affected uptake similarly, with fibroblast derived beta-interferon being more effective in dose response than gamma or alpha interferons. This suggests that endogenous production of interferon by cultured cells, such as that observed during a low grade viral infection, inhibits queuine uptake and may subsequently lead to a decreased level of queuine modified transfer RNA. Queuine-hypomodified transfer RNA has been implicated in growth control, differentiation and neoplastic transformation.

The queuosine modification of mammalian transfer RNA (tRNA) occurs as an irreversible post-transcriptional exchange of queuine base for the primary transcript guanine in the first position of the anticodon of tRNA. The reaction is catalyzed by the enzyme, tRNA-guanine ribosyltransferase, and is specific for tRNA isoacceptors which accommodate the amino acids, asparagine, histidine, tyrosine and aspartic acid (1-4). Queuine is not synthesized by mammalian cells and must be obtained through the diet or gut flora (5,6). Mammalian cells grown in culture must obtain queuine from the animal sera used to supplement the growth media (3,7).

The biochemical or functional significance of queuine remains largely unknown; however, transfer RNA isolated from neoplastic tissue is known to be queuine-hypomodified to various degrees (2,8,9). Furthermore, the degree of hypomodification has been associated with the severity of disease in human leukemias and lymphomas (10). In addition, when murine erythroleukemia cells were induced to differentiate, a significant increase in the queuosine content of tRNA was reported (11). The induction of differentiation

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and the increase in queuosine content of tRNA were both effectively blocked by the tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA).

Our data has demonstrated that chronic exposure of the phorbol ester tumor promoter, phorbol-12,13-didecanoate (PDD), to cultured human fibroblasts induces a transient inhibition of queuosine modification in tRNA (12). The decrease in queuosine content of tRNA always preceded an increase in the cell population density in culture. In later passage cultures, as queuosine levels began to be reestablished, the population densities came back down to near normal levels,indicating that queuosine levels and cell density were inversely related. Simultaneous addition of exogenous queuine with PDD blocked the tumor promoter-induced increase in population density and maintained the tRNA in a queuosine modified form.

Studies were initiated to analyze PDD effects on queuine uptake into cultured human fibroblasts. Uptake studies were performed with rQT_3, a radiolabelled analog of queuine that has the same affinity for the cellular uptake mechanism (13,14). The uptake mechanism for rQT_3 demonstrated biphasic kinetics with Kms of 350 nM and 30 nM. In general, most of the early passage human fibroblast cell cultures demonstrate an increase in rQT_3 uptake rates in the presence of PDD and other protein kinase C agonists (15, data herein). However, in a some early passage fibroblast cell cultures, PDD and a structurally unrelated stimulator of protein kinase C, teleocidin, were both capable of inducing inhibition of the high affinity component for queuine uptake (14). When conditioned media was transferred from an early passage phorbol ester-inhibition-sensitive cell culture to an insensitive cell culture, the culture was rendered susceptible to phorbol-induced inhibition of rQT_3 uptake (14). A protein factor in the media was identified that was stable when frozen at -20'C, heat inactivated at temperatures of 60'C and above, sensitive to treatment with proteases, and demonstrated an apparent molecular weight between 10 and 30 KDa by size exclusion membrane filtration (14). All attempts to mimic conditioned media factor with commercially available growth factors such as epidermal growth factor or platelet derived growth factor, resulted in the increase of rQT_3 uptake into cultured fibroblasts (14,15). However, polyinosinic-polycytidylic acid (poly-IC) potentiated sensitivity to PDD-induced inhibition of rQT_3 (14). Since poly-IC is a mimicker of viral infection and is routinely used to induce cellular responses consistent with viral infection including the induction of

interferon activity (16,17), we embarked on this study to analyze PDDs relationship to poly-IC and interferon as modulators of queuine uptake.

METHODS AND MATERIALS

Materials: Cell cultures were established and maintained in neonatal calf serum supplemented minimum essential media (MEM, GIBCO Grand Island NY). Media was further supplemented with 25mM HEPES buffer, 1X sodium pyruvate, 2X non-essential amino acids, 1X vitamins, penicillin-streptomycin, 0.2% sodium bicarbonate and the additional amino acids, asparagine, histidine, tyrosine and aspartic acid as previously described (13,14). The radiolabelled analog of queuine, rQT_3, was a gift from Dr. Jon R. Katze (University of Tennessee, Memphis). The compound was prepared from queuine by catalytic reduction and exchange as performed by Amersham (Arlington Heights IL). Lyophilized rQT_3 was dissolved in water to yield a stock concentration of 1 mM with a specific activity of 0.19 uCi/ug, and stored as a 200X stock solution at -20'C. Phorbol-12,13-didecanoate (PDD) was obtained from Sigma Chemical Company (St.Louis MO) and dissolved into acetone to a stock concentration of 1 mM. All PDD solutions were wrapped in foil to protect them from light and stored at -20'C. Polyinosinic-polycytidylic acid (poly-IC), human alpha and gamma interferons were purchased from Sigma Chemical Company. Human beta interferon and antibodies against human beta interferon were purchased from Alpha Therapeutics Corporation (Los Angles CA). All these factors were established as stock solutions in supplemented growth media and stored at -20'C.

Methods: Human skin cell cultures were established from neonatal foreskins using the method of Riegner et. al. (16), which results in a predominately fibroblast culture. Cell cultures were maintained in 25 cm^2 flasks with 5 ml of supplemented MEM containing 10% calf serum. Confluent cells were trypsinized and passaged at a 1 to 4 ratio into new flasks.

For assay or rQT_3 uptake , fibroblast were subcultured into 35 mm dishes at a density of $4x10^4$ cells/ml in a total volume of 2 ml of serum supplemented media. When cells reached confluency, the media was exchanged for 1 ml of fresh serum supplemented media containing 100 nM rQT_3. Uptake of rQT_3 into untreated control cultures was compared to cultures pre-treated with various amounts of poly-IC, alpha, beta or gamma interferon. Incubations were terminated by rinsing the cell sheet four times with 5 ml of ice-cold phosphate buffered saline. The cells were then lysed for five minutes under 1 ml of 95% ethanol, and the lysate aspirated and analyzed for rQT_3 by liquid scintillation.

RESULTS

Confluent early passage human fibroblasts were treated for 48 hours with 25 ug/ml of poly-IC. Following preincubation of the cells with poly-IC, rQT_3 uptake was measured either with or without 10 nM PDD at various time points for up to three hours (Figure 1). PDD exposure commonly resulted in stimulation of rQT_3 uptake to approximately 116% (sd=4%, n=4 separate fibroblast cultures,triplicates for each time point). Poly-IC treatment alone

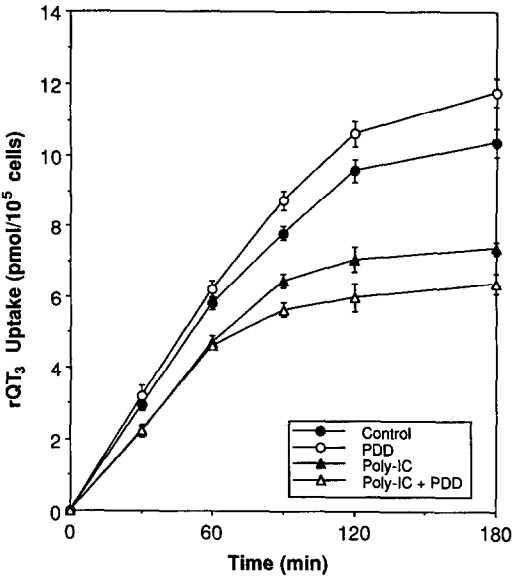

Figure 1. The effect of poly-IC on rQT_3 uptake. Confluent cell cultures in 35 mm dishes were divided into two groups. One group was treated with 25 ug/ml of poly-IC supplemented growth media for 48 hours while the other was left in normal growth media. The cultures were then treated for various time increments with 100 nM rQT_3 containing media, with or without 10 nM PDD. Symbols represent control (●), 10 nM PDD (○), poly-IC (▲), and poly-IC with PDD (△). Points represent the mean and standard deviation from triplicate assays.

inhibited uptake to approximately 70% (sd=10%, n=4 separate fibroblast cultures, triplicate assays at each time point). Combined PDD and poly-IC treatment resulted in a greater decrease in the uptake rate from that observed with poly-IC treatment alone (Figure 1).

Since one of the responses to poly-IC exposure is the induction of interferon and interferon-related systems, we analyzed human alpha, beta and gamma interferons for effects on rQT_3 uptake in confluent human fibroblast cell cultures. All interferons tested inhibited rQT_3 uptake to various extents. Furthermore, concurrent treatment of cultures with 10 nM PDD potentiated the inhibition of rQT_3 uptake at lower interferon concentrations.

Gamma-interferon (1000 IU/ml) was introduced to confluent fibroblast cultures for 1, 3, 6, 9, 12, 24, 36 and 48 hours prior to the addition of 100 nM rQT_3 with or without 10 nM PDD. Figure 2 demonstrates that cells treated with gamma-interferon alone did not inhibit rQT_3 uptake until after 24 hours of preincubation. Inhibition of rQT_3 was observed between 24 and 48 hours after preincubation with interferon. Additional exposure of the cells to interferon up to 72 hours did not appreciably enhance the

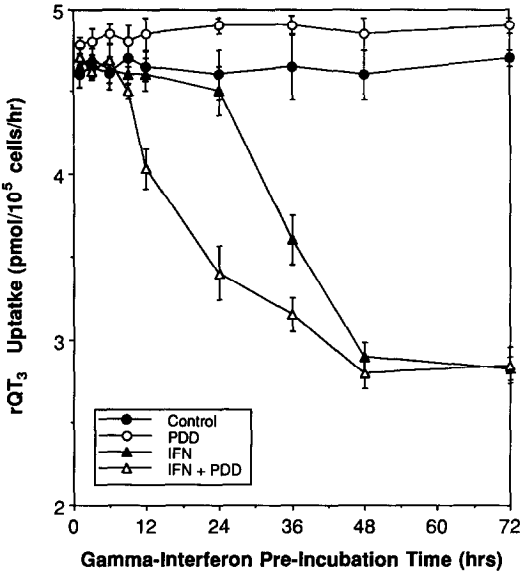

Figure 2. The effect of pre-incubation time with gamma-interferon on rQT_3 uptake. Confluent cell cultures in 35 mm dishes were divided into four groups. Two groups were treated with gamma-interferon for various time interval up to 72 hours. After pre-incubation the cells were exposed to 100 nM rQT_3 with or without 10 nM PDD, and the initial rate of uptake was determined as pmol/10^5 cells/hr. Symbols represent; control (●), 10 nM PDD (○), gamma-interferon treated (▲), and interferon with PDD (△). Points represent the mean and standard deviation from triplicate assays.

inhibition of rQT_3. Addition of 10 nM PDD to the incubation media shifted inhibition of rQT_3 uptake induced by interferon to between the 12 to 24 hour time frame, a period where interferon itself typically demonstrated no effects. Furthermore, although PDD alone acted to marginally stimulate rQT_3 uptake, PDD potentiated gamma-interferon induced-inhibition of rQT_3 at the 48 hour time point (Figure 2).

Similar results were observed with alpha and beta interferon pretreatment time courses. If equal molar concentrations of monoclonal antibody against beta-interferon was added concurrently with beta-interferon (100 IU/ml) for a 24 or 48 hour preincubation, then interferon induced inhibition of rQT_3 was partially blocked yielding an uptake of 90% of control instead of 50 to 60% of control. Antibody against beta-interferon also blocked poly-IC induced inhibition of rQT_3 uptake to a similar extent (data not shown). Addition of antibody 12 to 24 hours after the addition of interferon or poly-IC did not effect the inhibition of rQT_3 uptake.

A dose response curve was established for the three interferons inducing rQT_3 uptake inhibition (Figure 3). Maximal inhibition of rQT_3 uptake was observed after 48 hours of

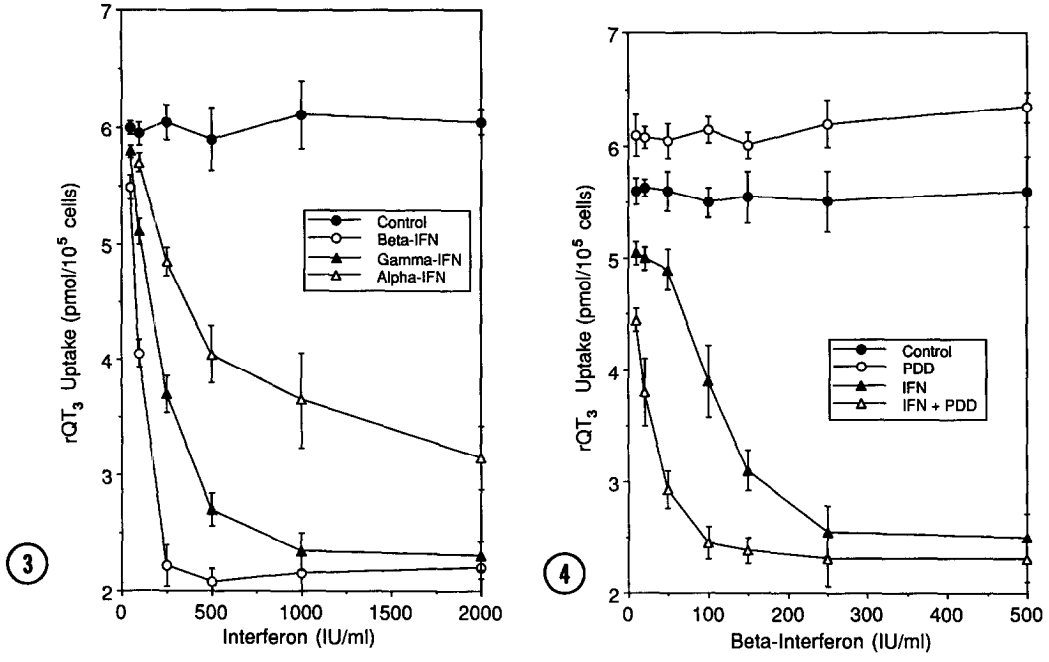

Figure 3. Dose response curve for alpha, beta and gamma-interferons on inhibition of rQT_3 uptake. Confluent cell cultures in 35 mm dishes were treated with interferons for 48 hours. The cells were exposed to 100 nM rQT_3 and initial rate of uptake was determined as pmol/10^5 cells/hr. Symbols represent; control (●), beta-interferon (○), gamma-interferon (▲), and alpha-interferon (△). Points represent the mean and standard deviation from triplicate assays.

Figure 4. The effect of 10 nM PDD in potentiating beta-interferon dose response inhibition of rQT_3 uptake. Confluent cell cultures in 35 mm dishes were divided into four groups, and two groups were treated with various concentrations of beta-interferon for 48 hours. Cells were then exposed to 100 nM rQT_3 with or without 10 nM PDD and initial rates of uptake were determined as pmol/10^5 cells/hr. Symbols represent control (●), 10 nM PDD treated (○), beta-interferon treated (▲), and interferon with PDD (△). Points represent the mean and standard deviation from triplicate assays.

preincubation with 250 IU/ml of beta-interferon, 600 IU/ml gamma-interferon and 2000 IU/ml alpha-interferon. Although individual responses are somewhat different, all interferons reduced rQT_3 uptake to a baseline level at approximately 40 to 50% of control.

Since beta-interferon is produced by fibroblasts during viral infections (16,17) we examined whether PDD could act together with sub-optimal doses of beta-interferon to induce greater inhibition of rQT_3 uptake. Again, PDD treatment alone acted to increase the rQT_3 uptake levels over that of untreated controls (Figure 4). Beta-interferon at 10 and 20 IU/ml induced minor inhibition of rQT_3 uptake; however, concurrent treatment with 10 nM PDD showed a major induction of uptake inhibition at the 10 and 20 IU/ml concentrations. PDD and interferon together demonstrated greater

induction of uptake-inhibition than interferon alone at sub-optimal levels. PDD could not further potentiate the inhibition of rQT_3 uptake at higher levels of interferon treatment (above 250 IU/ml).

Cell densities and viability were assessed for both treated and untreated cultures, and no significant differences between control and 72 hour interferon-treated cultures were observed. This suggests that rQT_3 uptake inhibition induced by interferon was not related to inhibition of cell growth or toxicity within our time frame of analysis. However, prolonged treatment with interferon (10 to 14 days) was shown to significantly inhibit cell growth (data not shown).

DISCUSSION

Previous studies have identified a specific uptake mechanism for queuine and its radioactive analog rQT_3 (13,14). It was demonstrated that the phorbol ester tumor promoter, PDD, could induce inhibition of rQT_3 uptake into a few select cultures of neonatal human foreskin fibroblasts (14). Those cells that exhibited sensitivity to PDD did so only in conjunction with an endogenously produced protein factor. The conditioned media factor, when exposed to PDD-insensitive cells for periods of 24 to 48 hours, rendered the cells sensitive to PDD treatment and rQT_3 uptake was inhibited (14). Poly-IC was shown to mimic the "conditioned media factor" and induce inhibition of rQT_3 uptake(14). Those results have been repeated here in this study. Since poly-IC has been used to induce interferon activity in numerous experimental systems (16,17), we analyzed interferon's effect on rQT_3 uptake.

Human interferons alpha, beta and gamma all inhibited rQT_3 uptake into culture human fibroblasts. All interferons required a 24 to 48 hour preincubation time prior to running the uptake experiment in order to illicit maximal inhibition. This implies that interferon is controlling a secondary interferon-dependent effector system that may be modulating the queuine uptake mechanism's activity. Activation of this system by either poly-IC or beta-interferon can be blocked by the addition of anti-beta-interferon antibody.

A dose response curve established that the fibroblast derived beta-interferon was the most efficient at inducing queuine uptake inhibition with a optimal dose of approximately 250 IU/ml. Gamma

and alpha-interferons had optimal doses of 600 and 2000 IU/ml respectively. All interferons reduced rQT_3 uptake to a basal level of approximately 50% (+/-10%) of control uptake levels. Furthermore, sub-optimal doses of interferon could have their inhibitory capability potentiated by the addition of 10 nM PDD.

Recently, the effects of human beta and gamma-interferon on protein kinase C were investigated (18). Treatment of two tumor cell lines with 1000 IU/ml of either interferon dramatically diminished protein kinase C activity. It was concluded that both beta and gamma- interferon induced long-term modulation of protein kinase C activity in tumor cells and that one effect of interferon treatment was to decrease activity of the enzyme. Additional studies have demonstrated copromoting activity of gamma-interferon in conjunction with 12-O-tetradecanoylphorbol-13-acetate (TPA) in multistage carcinogenesis of mouse skin (19). It was shown that low doses of intraperitoneally injected interferon increased the number of papillomas that developed after treatment with TPA, suggesting that interferon can systemically modulate TPA dependent promotion in mouse skin.

We conclude that interferon treatment inhibits queuine uptake in a time and dose dependent fashion and that sub-optimal doses of interferon can be potentiated to increased inhibition by concurrent treatment with PDD. Phorbol esters are direct stimulants of protein kinase C in the short term (20), and stimulate rQT_3 uptake (15, and data herein). However, chronic exposure of cells to phorbol esters has a down-regulating effect on protein kinase C activity (21). Since TPA and interferon have been shown to have co-promotion activity, and TPA and interferon have been shown to down-regulate protein kinase C activity, we propose that queuine uptake into cultured human fibroblasts is modulated by protein kinase C (15). Interferon and chronic exposure to phorbol esters will down-regulate protein kinase C and result in down-modulation of queuine uptake to a basal level. We now propose that the previously described "conditioned media factor" may be interferon. Furthermore, viral infection of cells leading to the endogenous production of interferon may lead to decreased availability of queuine in and around infected cells. The lack of sufficient substrate for tRNA-guanine ribosyltransferase could then result in queuine-hypomodified tRNA, which is a characteristic of transformed cells and has been implicated with tumor promotion activity.

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