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De novo protein synthesis is essential to human interferon γ gene expression by the stimulation with polyI:polyC

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Transcription of human interferon (IFN) γ gene is induced in human peripheral lymphocyte nylon-nonadherent cells (NNA cells) by double strand RNA poly I:poly C [(1985) J. Interferon Res. 5, 77–84]. In this report, the necessity of de novo protein synthesis in an early stage of IFN γ gene expression is described. For induction of IFN γ gene expression, only initial 4 h treatment of poly I:poly C to NNA cells is sufficient. Addition of inhibitor of protein synthesis, cycloheximide (CHX), at an early stage of induction periods (0–4 h) inhibits the IFN γ induction by poly I:poly C. Cell free translation assay using RNAs isolated from NNA cells which are induced by poly I:poly C in the presence of CHX reveals that in these RNAs, IFN γ mRNA does not exist. These results demonstrate that CHX inhibits de novo synthesis of a certain protein (or proteins) and for lack of the protein(s), IFN γ mRNA cannot be transcribed. The evidence is also described in this report which suggests that the essential protein(s) might be that (those) involved in protein kinase C (pkC) activation.

Interferon γ; Poly I:poly C; Gene expression; Cycloheximide; 12-*O*-Tetradecanoylphorbol 13-acetate

1. INTRODUCTION

Human IFN α, β, γ gene is controlled at the transcriptional level. These genes are transiently induced by various stimuli, such as double strand RNA or virus [2,3]. Among these three IFNs, IFN α and β resemble each other closely in their biological activities and cell surface receptors [4–6]. These two genes are both encoded in human chromosome 9 and possess very similar DNA sequences [7,8]. But IFN γ is rather different from IFN α/β in its biological activities and cell surface receptors [9,10]. Moreover, IFN γ is encoded in human chromosome 12.

About IFN α and β genes, especially about IFN β gene, the regulation of gene expression is fairly well studied [11,12]. But for human IFN γ gene expression, little is known about the regulation of its gene expression.

We previously reported that human IFN γ was induced by double strand RNA poly I:poly C [1]. Further, pkC and cyclic AMP dependent protein kinase are probably involved in induction of IFN γ by stimulation with poly I:poly C [13].

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Abbreviations: IFN, interferon; NNA cells, nylon-nonadherent cells; pkC, protein kinase C; PBML, peripheral blood mononuclear lymphocyte; cAMP, adenosine 3′,5′-cyclic monophosphate; CHX, cycloheximide; TPA, 12-*O*-tetradecanoylphorbol 13-acetate

In this report, we describe that de novo protein synthesis at an early stage of induction is essential to IFN γ gene expression by poly I:poly C. In the case that protein kinase C (pkC) activator 12-*O*-tetradecanoylphorbol-13-acetate (TPA) is present with poly I:poly C, of which great enhancement effect on IFN γ induction is already reported [14,15], the effect of CHX is more transient than poly I:poly C single stimulation. So the essential protein(s) to IFN γ gene expression might be the protein(s) involved in pkC activation.

2. MATERIALS AND METHODS

2.1. *Cell separation*

Human peripheral blood mononuclear lymphocytes (PBML) were isolated from whole blood of healthy donors by Ficoll hypaque (Pharmacia LKB Biotechnology) gradient centrifugation [16]. PBML at 2×10^6 cells/ml in RPMI 1640 medium supplemented with heat-inactivated fetal bovine serum (HyClone, Logan, UT) were incubated at 37°C for 60 min in tissue culture plastic dishes (Corning, 25010, NY), and nonadherent cells were carefully collected. NNA cells were separated by incubating cells not adhering to plastic dishes in a nylon column at 37°C for 60 min. After elution with the above medium, nonadherent cells were washed and collected for IFN γ induction.

2.2. *IFN production and titration*

NNA cells at 5×10^6 cells/ml in the above medium were treated with either poly I:poly C (PL Biochemicals, Milwaukee, WI) or phytohemagglutinin (PHA; Seikagaku Kogyo Co., Ltd). After incubation for 20–24 h (60 h in the case of PHA induction) at 37°C, supernatants were collected and assayed for IFN γ titer.

IFN γ assays were carried out in FL cells using Sindbis virus for challenging. IFN γ titers are expressed in international units, based on reference standards for human IFN γ (G-023-901-530) supplied by NIH.

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Cycloheximide (CHX; PL Biochemicals, Milwaukee, WI) was treated at the same time of inducer treatment. After several hours of incubation periods, CHX and inducers were washed out with PBS(−) and further incubated in fresh growth medium for 20–24 h.

TPA (Sigma) was simultaneously treated with poly I:poly C to NNA cell cultures.

2.3. *Measurement of ^3H incorporation*

NNA cells were inoculated in 96 well multi plates ($1–2 \times 10^6$ cells/0.1 ml/well) and pulse labeled for 20 h with 37 kBq/well of [^{3}H]thymidine (74 GBq/mmol), [^{3}H]uridine (5661 GBq/mmol) or [^{3}H]leucine (925 GBq/mmol) (New England Nuclear). Then the ^3H uptake into high molecular weight materials was trapped on glass fiber filters and the radioactivities were counted in a liquid scintillation counter.

2.4. *RNA preparation*

Total RNA was prepared from stimulated NNA cells with poly I:poly C or PHA with or without CHX, according to acid-guanidinium-thiocyanate-chloroform methods described by Chomczynski et al. [17].

2.5. *Cell free translation*

Cell free translation of isolated RNA was processed at 30°C for 4 h using rabbit reticulocyte cell free translation system (Boehringer Mannheim GmbH) in the presence of [^{14}C]methionine (2.07 GBq/mmol; Amersham International plc).

2.6. *Immunoprecipitation of IFN γ*

Immunoprecipitation of IFN γ protein was carried out for 1 h at 37°C and overnight at 4°C with rabbit polyclonal anti-human γ IFN serum (Genzyme, England). Precipitation of immune complexes was accomplished by addition of 100 μl of a 10% solution of *Staphylococcus aureus* (Pansorbin; Calbiochem). After 30 min incubation periods, *S. aureus* cells were washed extensively with a buffer containing 0.02 M PO_4 pH 6.8, 0.15 M NaCl, 0.5% Nonidet P-40, 5 mg/ml bovine serum albumin. The proteins bound to *S. aureus* were solubilized by boiling in 50 μl 0.05 M Tris-HCl pH 6.5, 2% SDS, 5% 2-mercaptoethanol, 20% glycerol.

2.7. *SDS-polyacrylamide gel electrophoresis*

Labeled IFN γ was resolved by electrophoresis in 10% polyacrylamide slab gels in the presence of SDS essentially as described by Laemmli et al. [18]. After staining and destaining, gels were dried and enhanced with EN^{3}HANCE (DuPont, USA), then exposed to Kodak X-ray film XR-2.

3. RESULTS

Dose dependence of CHX inhibition of IFN γ induction is shown in fig.1. Even low concentrations of CHX (0.05–0.1 μg/ml) could well inhibit IFN γ induction by poly I:poly C. On the other hand, over all proteins, DNA and RNA synthesis was only a little inhibited in these doses of CHX.

Time course of CHX inhibition is shown in fig.2. With 3–4 h delayed treatment after poly I:poly C stimulation, the addition of CHX showed no more inhibitory effect on IFN γ induction. Earlier treatment of CHX (0–3 h after poly I:poly C addition) greatly inhibited IFN γ induction.

It was feared that the long treatment of CHX (fig.2, 0–5 h) might damage overall cell function, resulting in inhibition of IFN γ induction. To eliminate this possibility, we treated CHX to cells at each time point

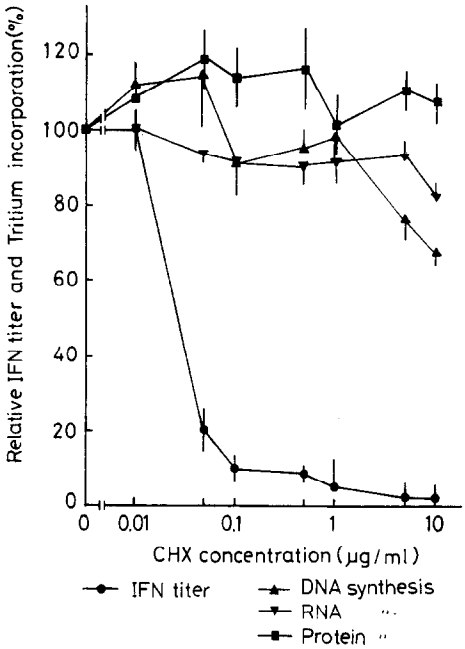

Fig.1. Dose responses of CHX inhibition on DNA, RNA and protein synthesis and IFN γ induction; NNA cells were stimulated with 100 μg/ml of poly I:poly C in the presence of above indicated doses of CHX for 4 h. After washing of poly I:poly C and CHX, cells were suspended in fresh growth medium and inoculated into 96 well plates, then pulse labeled for 20 h with [^{3}H]thymidine, [^{3}H]uridine and [^{3}H]leucine as indicated in section 2. The 24 h cultured supernatants were assayed for IFN γ titer. Data are expressed as the percentage of each IFN γ titer and tritium incorporation (cpm).

for only 2 h. The results were shown in fig.3. These results well accorded with the results of fig.2 concerning time course. That is to say, 3–5 h delay of treatment of CHX after poly I:poly C addition expressed

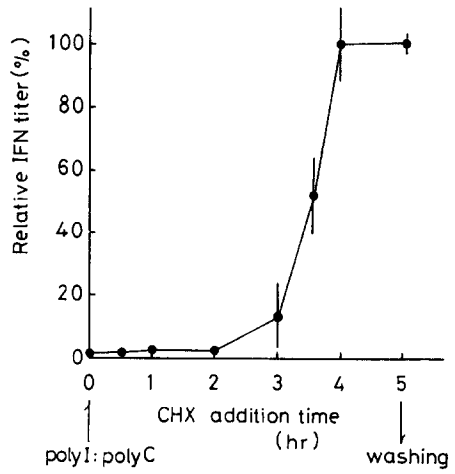

Fig.2. Time course of CHX inhibition after poly I:poly C addition; NNA cells were induced by 100 μg/ml of poly I:poly C (0 h). Then CHX (1 μg/ml) was added to the culture at 0–5, 0.5–5, 1–5, 2–5, 3–5, 3.5–5, 4–5 h. At 5 h after poly I:poly C addition, all cultures were washed and resuspended in fresh growth medium. After further incubation for 20 h, supernatant IFN γ titers were assayed. Data are expressed as percentage of each IFN γ titer.

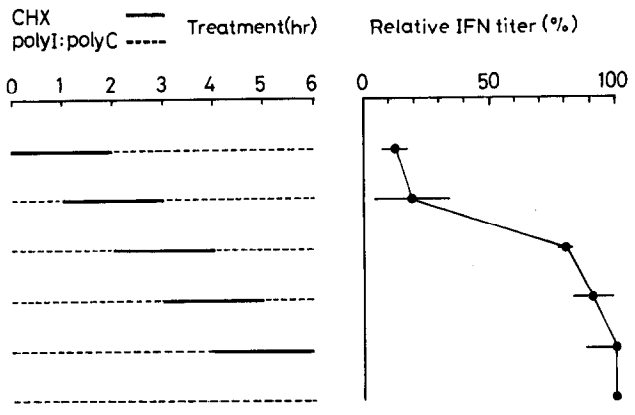

Fig.3. Time course of CHX inhibition after poly I:poly C addition. NNA cells were induced by 100 μg/ml of poly I:poly C (0 h) and CHX was added to the cultures at 0–2, 1–3, 2–4, 3–5, 4–6 h respectively as indicated in (left). At 6 h after poly I:poly C addition, the cultures were washed and resuspended in fresh growth medium and further incubated for 20 h. After incubation supernatant IFN γ titers were assayed. Data are expressed as the percent of each IFN γ titer (right).

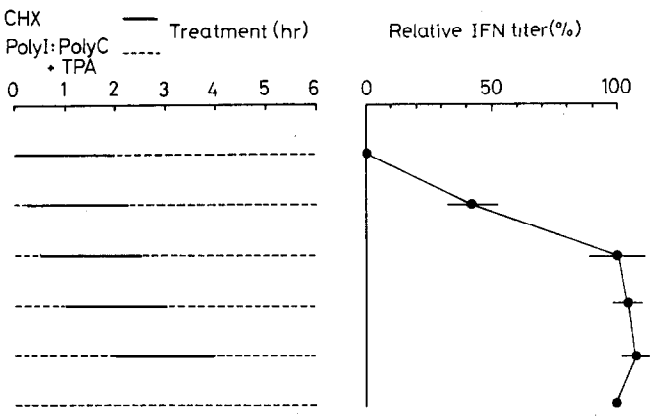

Fig.5. Time course of CHX inhibition after poly I:poly C addition in the presence of TPA. NNA cells were induced by 100 μg/ml of poly I:poly C in the presence of 10 ng/ml of TPA. CHX was added to the culture at 0–5 h; 15 min–5 h, 30 min–5 h, 1–5 h, 1.5–5 h, 2–5 h, 3–5 h, 4–5 h. At 5 h after poly I:poly C addition, all cultures were washed and resuspended in fresh growth medium and further incubated for 20 h. After incubation was over, supernatant IFN γ titers were assayed. Data are expressed as the percent of each IFN γ titer.

no more inhibitory effect. From these results, it was suggested that de novo protein synthesis was necessary at an early stage of IFN γ induction by poly I:poly C.

So we examined whether IFN γ mRNA was synthesized in NNA cells which were stimulated with poly I:poly C in the presence of CHX. For this, we isolated RNAs from various stimulated NNA cells, then using these isolated RNAs cell free translations were performed. The cell free translation products were then processed in radioimmunoprecipitation assay for IFN γ proteins. Fig.4 shows the fluorogram of radioimmunoprecipitation materials. From these results, it was proved that in the RNAs isolated from NNA cells which were stimulated by poly I:poly C in the presence of CHX scarcely any IFN mRNA existed. On the other hand, the RNAs isolated from NNA cells stimulated by PHA in the presence of CHX contained much more mRNA of IFN γ than those of NNA cells stimulated by PHA in the absence of CHX.

From these results, we concluded that, when NNA cells were stimulated with poly I:poly C for IFN γ production, de novo protein synthesis at an early stage of induction was essential to IFN γ mRNA transcription.

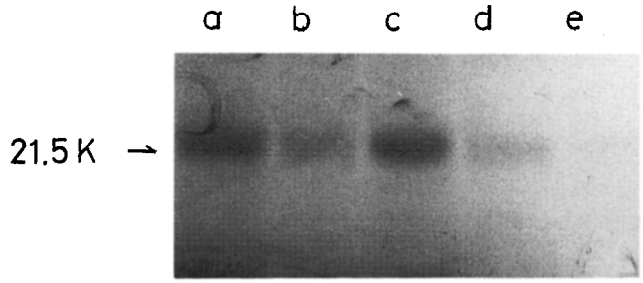

Fig.4. Fluorograph of ^{14}C-labeled radioimmunoprecipitation materials. The sources of mRNA in cell free translation are NNA cells stimulated with (a) 10 μg/ml of PHA, (b) 100 μg/ml of poly I:poly C, (c) 10 μg/ml of PHA in the presence of 1 μg/ml of CHX, (d) 100 μg/ml of poly I:poly C in the presence of 1 μg/ml of CHX, (e) none, for 2 h at 37°C. Cell free translation and immunoprecipitation were performed as described in section 2. The immunoprecipitation materials were processed in SDS polyacrylamide-gel-electrophoresis and fluorography.

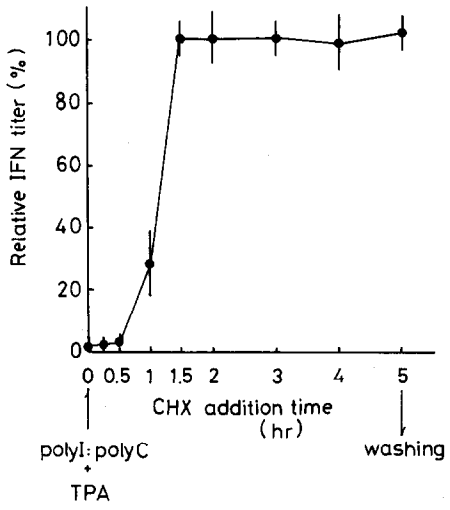

Fig.6. Time course of CHX inhibition after poly I:poly C addition in the presence of TPA. NNA cells were induced by 100 μg/ml of poly I:poly C in the presence of 10 ng/ml of TPA. CHX was added to the cultures at 0–2 h, 15 min–2 h 15 min, 30 min–2 h 30 min, 1–3 h, 2–4 h, respectively, as indicated (left). At 6 h after poly I:poly C addition, the cultures were washed and resuspended in fresh growth medium and further incubated for 20 h. After incubation was over, supernatant IFN γ titers were assayed. Data are expressed as the percent of each IFN γ titer.

Though it was not clear what protein was essential to IFN γ gene expression by poly I:poly C, we supposed that the proteins involved in pkC activation might be indispensable, because we had previously found that pkC was probably involved in induction of IFN γ by stimulation with poly I:poly C alone [13]. When NNA cells were induced by poly I:poly C in the presence of TPA, a protein kinase activator, the CHX also inhibited the IFN γ production only if it was added to the culture at a fairly early stage of induction (fig.5). But the time course of disappearance of the inhibitory effect was faster than that of poly I:poly C single stimulation (compare fig.2). Moreover, the experiments of addition of CHX for limited time also showed that 0.5–1 h delayed addition of CHX after poly I:poly C stimulation eliminated the inhibitory effect on IFN γ induction in the presence of TPA (fig.6).

4. DISCUSSION

We have demonstrated that de novo protein synthesis was essential at an early stage of IFN γ gene expression by poly I:poly C in human lymphocyte NNA cells.

In other cases of gene expression with various stimuli, de novo protein synthesis is not always necessary to gene expression [19]. In the case of IFN β induction in human diploid fibroblast, CHX treatment rather enhanced IFN β induction. This phenomenon is explained as superinduction [20,21].

In human lymphocyte NNA culture, IFN γ induction by PHA was not inhibited or even enhanced with CHX treatment (data not shown). But in the case of IFN γ induction by poly I:poly C, the induction was completely inhibited by the CHX treatment at an early stage of induction. This difference might be referred to the time lag between two inducers about the ability of bringing the saturated level of IFN γ titer. (In PHA induction, maximal IFN γ titer was obtained at 48–60 h after stimulation. In poly I:poly C induction, it was obtained 6–8 h later.) And this time lag might have resulted from difference of signal transduction mechanisms involved in IFN γ gene expression between these two inducers.

From the experiment of using TPA, it was strongly suggested that the protein(s) involved in pkC activation might be essential to poly I:poly C induction. It was proved to be essential to IFN γ gene expression that the protein(s) might be de novo synthesized by poly I:poly C stimulation in advance of IFN γ gene expression.

It was also suggested that the turnover of the protein(s) was rather transient, because 3–4 h delayed addition of CHX after poly I:poly C stimulation had no more inhibitory effect on IFN γ induction. A hypothesis about signal transduction and gene expression is the following: When NNA cells are stimulated with poly I:poly C, the signals are transduced to enhance de novo protein synthesis, and these proteins that are probably involved in pkC activation result in high level of protein kinase C activation and bring about IFN γ gene expression.

When TPA is present with poly I:poly C, owing to the effect of TPA, activation of pkC is induced up to a level almost sufficient for IFN γ gene expression at a relative early stage of induction, so that at a much earlier time compared to poly I:poly C single stimulation, IFN γ gene expression is completed.

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