

# IN VITRO SEMI-QUANTITATIVE DETERMINATION OF HUMAN GAMMA-INTERFERON EXPRESSION BY RT-PCR

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**Abstract-** Cytokines secreted by Th1 (T-helper)/Th2 cells play an important role in the pathogenesis of many diseases. Th1 cells secrete predominantly IFN- $\gamma$  and IL-2 which regulate cell-mediated immunity against intracellular pathogens and tumors. In this study, expression of IFN- $\gamma$  was studied using semi-quantitative real time polymerase chain reaction (RT-PCR). In brief, lymphocytes of a healthy donor were stimulated with PHA (1 $\mu$ g/10<sup>6</sup> cell/ml) in cell culture at different incubation times (0, 4, 8, 12, 24, 48 and 72 hours) to express IFN- $\gamma$ . Total RNA was extracted and cDNA synthesized. A sequence (273 bp) between two oligonucleotide primers (chosen from two different exons of the IFN- $\gamma$  gene sequences) was amplified using a heat-stable DNA polymerase. In semi-quantitative RT-PCR, we used a serial dilution for cDNA in order to determine the titer of cDNA which gives visible band in agarose gel (2%) electrophoresis. Results showed that the highest level of IFN- $\gamma$  expression was achieved after 4 hours activation with PHA and it was stable at least for 22 hours. Then it fell to baseline level. Cytokine detection using RT-PCR will provide useful clinical information.

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**Key words:** RT-PCR, semi-quantitative, IFN- $\gamma$ , phytohaemagglutinin

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## INTRODUCTION

Cytokines of T-helper (Th)1/Th2 cells play an important role in the pathogenesis of some diseases. Th1 cells secrete predominantly IFN- $\gamma$  and IL-2 which regulate cell-mediated immunity against intracellular pathogens and tumors. So, different expression of IFN- $\gamma$  which runs cellular and inhibits humoral immune system can cause many diseases (1, 2). IFN- $\gamma$  is a homodimeric glycoprotein consisting of two 21 to 24 Kd subunits. The size variation of the subunit is caused by variable degrees of glycosylation, but each subunit contains one identical 18 KD

polypeptide encoded by the same gene. The single IFN- $\gamma$  gene is located on chromosome 12. This gene includes four exons and three introns. There is also low polymorphism in the gene (3, 4).

In general, to quantitative detection of cytokines expression, there are several methods including: bioassay, determining functions and actions of cytokines in cell culture (5), protein assay, detection of total protein of cytokines in a solution by enzyme linked immunoadsorbent assay (ELISA) and radioactive immune assay (RIA) tests (6) and message assay, currently a gold standard method which can be used to detect mRNA of cytokines (7). Because Taq polymerase is unable to amplify the mRNA as template, for quantitative detection of IFN- $\gamma$ -mRNA, it is reversed transcribed to make cDNA which will be amplified by real time polymerase chain reaction (RT-PCR) (8-12). A simple method for application of quantitative RT-

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## Semi-quantitative determination of human IFN- $\gamma$

PCR is making serial dilutions from DNA or cDNA and performing PCR by these serial dilutions. Then select the final dilution which gives visible band in agarose gel (2%) electrophoresis as titer of DNA or cDNA (9).

RT-PCR provides sensitive and accurate method and can also be used for clinical sample because they are almost small samples (13-15). We investigated the optimum time required for high level expression of IFN- $\gamma$  by lymphocytes stimulated with phytohaemagglutinin (PHA) in cell culture. Obtaining this time can be used to detect the effects of various agents like viruses and drugs on IFN- $\gamma$  gene expression. In addition, it can be useful in cloning and expression of IFN- $\gamma$  in eukaryote cells.

## MATERIALS AND METHODS

### Lymphocyte isolation and stimulation with PHA

Ten ml sterile blood was drained from a healthy donor and mixed by 1 ml 10% EDTA and diluted with 22 ml Hank's solution. Peripheral blood lymphocytes (PBLs) were isolated by Ficoll and washed two times with Hank's solution. About  $6 \times 10^6$  lymphocytes were suspended in 12 ml RPMI1640 medium (Gibco-BRL, Australia) containing 100 u/ml penicillin G (Hayan, Iran), 20% FCS (Gibco-BRL, Australia), 100  $\mu$ g/ml streptomycin (Hayan, Iran) and 10  $\mu$ g/ml PHA (Sigma, Germany), within six well plate and incubated in different times (0 h, 4 h, 8 h, 12 h, 24 h, 48 h and 72 h) in CO<sub>2</sub> incubator (9, 16).

### Total RNA extraction and single cDNA synthesis

Lymphocytes were harvested by centrifuge and (their viability were more than 80%) washed two times with Hanks' solution. Lymphocyte pellet suspended with 1 ml Trizole solution (containing acid gonidium thiocyanate/phenol) and 200  $\mu$ l chloroform solution centrifuged at  $12000 \times g$ . Two phase appeared; aqueous phase (upper) were isolated and total RNA precipitated by cold ethanol; RNA pellet was washed by 70% alcohol then suspended in 15  $\mu$ l DEPC (diethyl pyrocarbonate) treated water.

Amount of RNA were determined with 260 nm absorbance. All above procedures were done in 4°C (9, 16). One  $\mu$ g total RNA, 0.5  $\mu$ g oligo dT<sub>18</sub> primer were added to a microtube and incubated for 5 min in 70°C, cooled on ice. dNTP (1 mM), Tris buffer (10 mM), RNase inhibitor (40 u/20  $\mu$ l) were added and 5 min incubated in 37°C, finally 200 u M-MuLV enzyme was added to cocktail and incubated for 60 min in 37°C. Then incubated for 10 min in 70°C in order to inactivate the enzyme. Synthesized cDNA stored in -20°C (9).

### PCR

Each PCR reaction was performed in a total volume of 20  $\mu$ l containing, Tris buffer (10 mM, pH = 8 in 20°C), MgCl<sub>2</sub> (2 mM), dNTP mix (0.8 mM, each dATP, dTTP, dCTP, dGTP 0.2 mM), Taq DNA polymerase (0.5 u), cDNA (2  $\mu$ l), sense primer; 5'-AAT GCA GGT CATTCA GAT G-3' and antisense primer 5'-TTG GAC ATT CAA GTC AGT T -3' (each 0.5  $\mu$ M), product size 273 bp. An initial denaturation step of 5 min 94°C was followed by 40 cycles of: 45 sec 94°C, 60 sec 55°C, 60 sec 72°C, and end step 5 min 72°C (9).

### Semi-quantitative PCR

Different amount of total RNA was obtained during RNA extraction. We unified the volumes that contain exactly 1  $\mu$ g of RNA (13). Two fold dilution series (1/2, 1/4....) of cDNA were prepared in water for each incubation time, the aliquots were added to PCR reaction mix and PCR performed for each dilution. The final dilution which gives visible band on the 2% gel was recorded as incubation time titer (12, 16).

### Gel electrophoresis

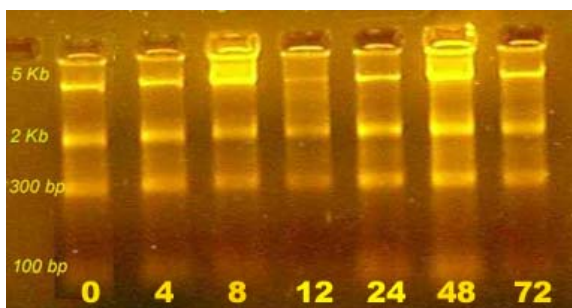
Agarose gel were prepared with TAE buffer (Tris, glacial acetic acid, EDTA, pH=8) and ethidium bromide was added (1  $\mu$ g/15 ml gel) to it. Five  $\mu$ l PCR product were mixed with 1  $\mu$ l of sample buffer (6X: 0.25% bromophenol, 0.25% xylene cyanol, 15% Ficoll 400) and loaded on 2% agarose and electrophoresed in 80 Volt for 25-30 min. Band of fragments were observed by UV transilluminator and documented by Kodak digital camera (9).

## RESULTS

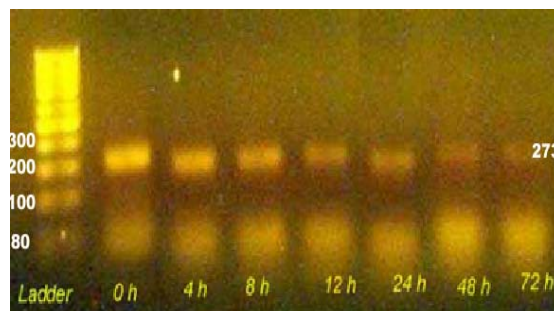
Four different distinct bands observed in the electrophoresis pattern of the total RNA which was extracted from incubated lymphocytes with PHA at different times. These bands from up to down were: 5Kb, 2Kb, 300bp, and 100bp which exactly correlate with eukaryotic cells RNAs 28S, 18S, 5S and tRNA, respectively (Fig. 1).

The electrophoresis of the RT-PCR products which were obtained from incubated lymphocytes with PHA at different times (without serial dilution) showed IFN- $\gamma$  gene expression even without incubation (0 h) of lymphocytes with PHA (Fig. 2). The electrophoresis of the RT-PCR products obtained from incubated lymphocytes with PHA at different times (with preparing serial dilution) showed 4 hours incubation of lymphocytes with PHA is enough and IFN- $\gamma$  gene expression is highest (titer 512, Fig. 3b). For other incubation times, titers were: 0 h = 16, 8 h = 64, 12 h = 64, 24 h = 64, 48 h = 32 and 72 h = 16 (Fig. 3).

To assay the amount of IFN- $\gamma$  gene in each incubation time of lymphocytes with PHA, we diluted each cDNA of each incubation times of lymphocytes (dilution rate: 1/2), then we performed RT-PCR for each dilution. The high expression of IFN- $\gamma$  gene was transient and rapidly came to background (Fig. 4). Results of semi-quantitative RT-PCR from lymphocytes which were incubated at different times with PHA with serial dilutions showed the highest expression of IFN- $\gamma$  gene occur after 4 hours activation with PHA.



**Fig. 1.** The agarose gel (2%) electrophoresis pattern of the total RNA which was extracted from lymphocytes incubated with PHA at different times. Observed bands containing: 5Kb, 2Kb, 300bp and 100bp which exactly correlate with eukaryotic cells RNAs 28S, 18S, 5S and tRNA, respectively.



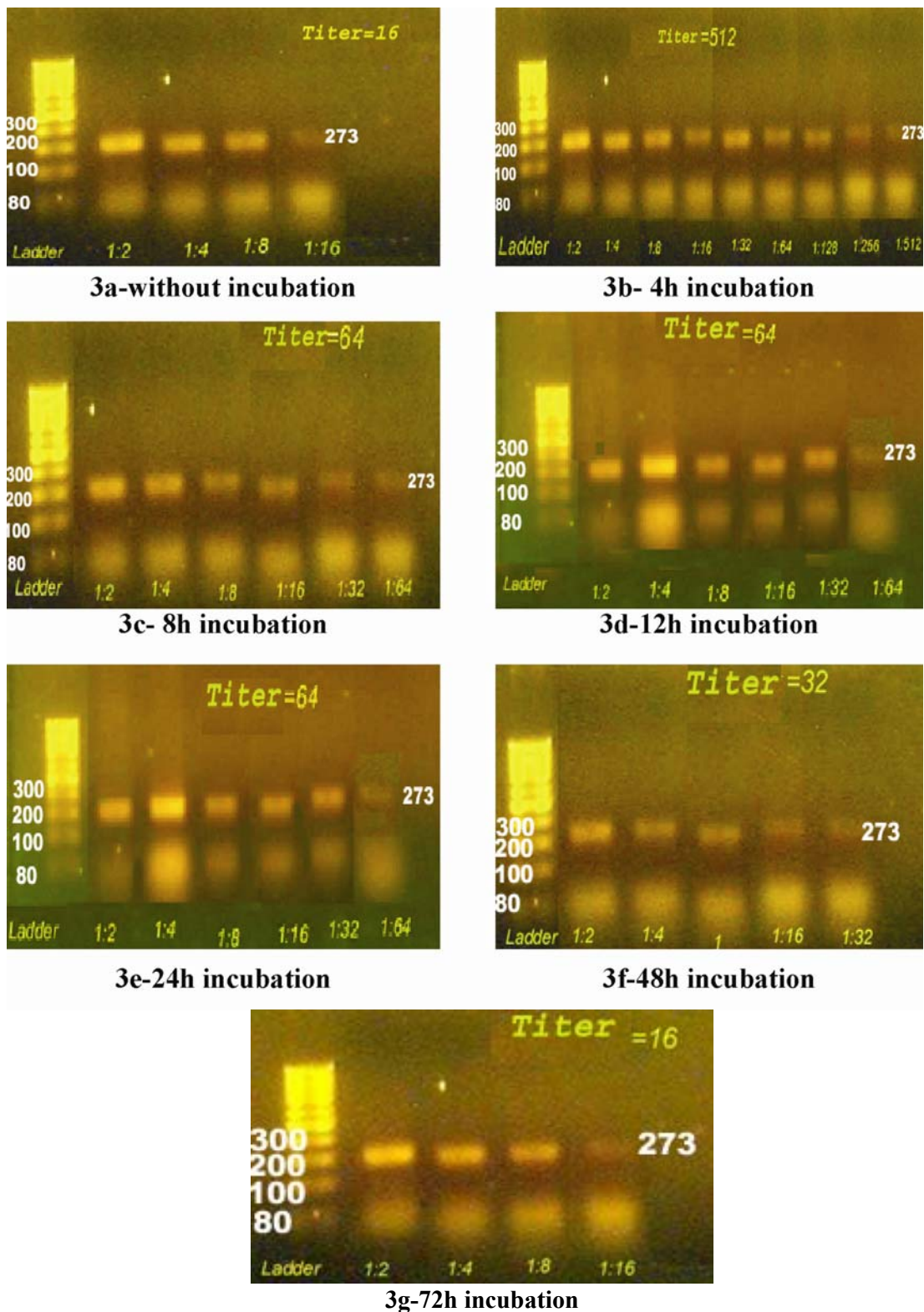
**Fig. 2.** The agarose gel (2%) electrophoresis pattern of the RT-PCR products which were obtained from lymphocytes incubated in different times with PHA (without serial dilution). Lane1, molecular size marker. Lanes 2, 3, 4, 5, 6 and 7 amplified fragment (273 bp) in different incubation times.

## DISCUSSION

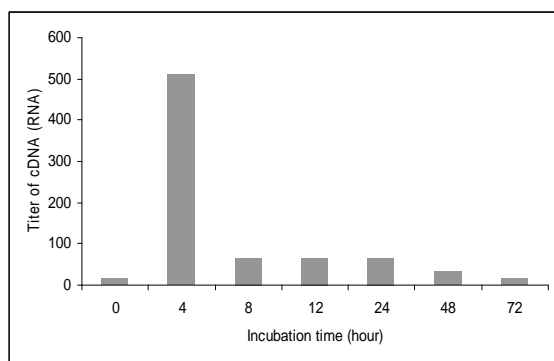
Recent data have suggested that the expression of cytokines and chemokines by lymphocytes could play an important role in the development of other adaptive immune responses. It is well established that the ability of T-helper lymphocytes to mediate many of their coactivating functions is through the rapidly production of soluble mediators such as IFN- $\gamma$  (17).

We developed a semi-quantitative RT-PCR assay (by serial dilution assay) to analysis expression of mRNA of the IFN- $\gamma$ . The assay showed that, four hours incubation of lymphocytes with PHA increased IFN- $\gamma$  expression to the highest level (Fig. 3b. titer 512). Strangely, measuring by RT-PCR showed IFN- $\gamma$  gene was expressed in lymphocyte culture supernatants even without incubation with PHA (Fig. 3a. titer 16). So, IFN- $\gamma$  expression decreased rapidly and reached to 64 which remained 22 hours stable, then it reached to background (like without stimulation with PHA) (Fig. 4). Meanwhile, because IFN- $\gamma$  mRNA is labile (7), the IFN- $\gamma$  expressed *in vitro* may not be entirely representative of *in vivo* expression but we can propose, in the body (*in vivo*), after elimination of invader (viruses, bacteria ...) or stimulant, IFN- $\gamma$  level decreases rapidly due to liability of its mRNA.

As we know, if it doesn't decrease, it will cause many autoimmune and allergic diseases (1). In a similar study by other researchers, they reported



**Fig. 3.** The agarose gel (2%) electrophoresis pattern of semi-quantitative RT-PCR for lymphocytes which were incubated at different times with PHA (serial dilution).



**Fig. 4.** The expression of IFN- $\gamma$  in different times of incubation with phytohaemagglutinin (PHA). Incubation of lymphocytes with PHA stimulated rapidly the express of gamma interferon gene and reached to peak in 4 hours. Then it's level decreased and stabled for about 22 hours. After this period, it reached to the background.

similar expression patterns for IFN- $\gamma$  measuring by ELISA. But the titer of IFN- $\gamma$  in lymphocytes without incubation with PHA was about zero (16).

This inconsistency with our data can be due to that ELISA method is not much sensitive as RT-PCR and can not detect small amount of cytokines proteins. In addition, gene expression data provide an understanding of gene regulation in a specific biological process at the transcriptional level. However, protein detection methods such as ELISA determine whether the transcribed gene is ultimately translated to protein. Also, lymphocytes of some healthy people can express IFN- $\gamma$  without stimulation in cell culture (8, 13, 18, 19). Other researchers have also reported other cytokines expression patterns similar to IFN- $\gamma$ . Sometimes these reports are controversial. We think this may be correlated to different lymphocyte culture conditions, detection methods and kind of the cytokines (13-16, 20).

In conclusion, we believe that cytokine detection using RT-PCR will provide useful clinical information between different cytokines and disease progression.

### Conflict of interests

The authors declare that they have no competing interests.

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