A Simple ELISA System for Detection of hIFN-γ

Short Communication

Zeinoddini, M., Maghsoudi*, N., Akbari, B., Mohammadi, M.R., Dèldar, A.A. and Sadeghi, V.

Genetic Engineering Dept., Research Center for Sciences and Biotechnology, Malek Ashtar Industrial University, P.O.Box 19395-1949, Tehran, Iran

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Summary

The use of accurate, rapid, cheap and sensitive methods for measurement of cytokines in body fluids is absolute prerequisite to define involvement of these mediators in various clinical situations and pharmacological effect of recombinant cytokines administration. In this study a sandwich enzyme-linked immunosorbent assay (ELISA) was designed for detection of small amounts of human interferon gamma (hIFN-γ). After immunization of a female New Zealand White (NZW) rabbit and a female BALB/c mouse against recombinant human IFN-γ, a high level titer of antibody was produced that confirmed by dot blot technique. A precipitated antibody from rabbit serum was used as first antibody and mouse serum used as second antibody. By determination of the best antibody concentrations and optimization of other conditions, an ELISA system was designed. The data indicate that this ELISA was efficient and sensitive for detection of as little as 40ng/ml of recombinant hIFN-γ.

Key words: ELISA, hIFN-γ, polyclonal antibody

Introduction

IFN-γ is a lymphokine, produced by activated T-cells. This molecule is involved in a variety of important biological activities such as immune system regulation, and antiviral activity (Trinchieri & Perussia 1985). Since the first option in determination of biological activity of IFN-γ is its antiviral activity, nowadays the standard method for determination of IFN-γ is very essential (Altrock 1986).

*Author for correspondence. E-mail: maghsudi@yahoo.com

Different methods for detection of some cytokines have been proposed during last years (Granger *et al* 1999). The base of these methods rely on monoclonal antibodies which are more sensitive and convinient than bioassay methods (Augsburger 2000). Since the level of IFN-γ in serum never increases to high levels and it acts locally, the determination of its concentration in serum needs highly sensitive methods. The bioassay of IFN-γ has an important application e.g.; it is an index for cellular immunity in some diseases like malaria (Schofield *et al* 1987). On the other hand use of high sensitive method is necessary in production of recombinant IFN-γ, where we deal with very small quantities. So a specific method with moderate expense and sensitivity could be a suitable method for determination of this protein. On the basis of recent studies we designed a simple method for determination of IFN-γ concentrating by means of the polyclonal antibody that produced in immunized rabbit and mouse.

Materials and Methods

Polyclonal antibody production in rabbit. A 2 to 3-month-old New Zealand White (NZW) rabbit immunized by injection of rhIFN-γ (Imukin–Boehringer). Pre-immunization sera collected as negative control. Then 100μg of IFN-γ mixed completely with 0.5ml of PBS (pH7.4) and 0.5ml Freund's complete adjuvant and injected subcutaneously into local outbreed rabbit. After one month, the serum of rabbit was collected and then 50μg of antigen was mixed with 0.5ml of PBS and 0.5ml Freund's incomplete adjuvant and injected as first booster.

The same process repeated as second and third boosters with two-week intervals. Finally after approaching to a high titer of anti rhIFN-γ antibodies the animal was bled and it's serum pooled and stored at -20°C until use.

Precipitation of polyclonal anti-IFN-γ antibody by ammonium sulfate. About 5ml serum from immunized rabbit mixed with 5ml of saturated ammonium sulfate. After completely mixing, the mixture was kept at 4°C for 4-5h then centrifuged in

8000rpm for 20min at 4°C. After removing the supernatant, 1ml of PBS was added to the pellet and suspended. In the next step, the suspension dialyzed two times against PBS with a membrane of 10-12Kda pore size dialysis bag and the concentration determined with spectrophotometer at 230, 260 and 280nm.

Polyclonal antibody production in mouse. Two 8-week-old BALB/c mice bled for collection of negative control serum. They immunized of a mixture of 50μg rhIFN, 250μl PBS and 250μl Freund's complete adjuvant subcutaneously. After one month a mixture of 25μg antigen, 250μl PBS and 250μl Freund's incomplete adjuvant was injected same as the first booster. The second and third boosters also injected at two weeks intervals. After confirmation of the high antibody titer by dot blotting technique, the mice bled and their sera pooled and stored at -20°C.

Dot blot technique for determination of antibody titer. At the first, the PolyVinylidene Difluride (PVDF, Roche Co.,) membranes were prepared in relevant dimensions and proccesed as the follow: 1) for a few second in pure methanol 2) for a few minutes in distilled water 3) for a few minutes in transfer buffer (Tris 20mM, Glysin 15mM, 20% methanol, pH8). Then 50μl of 10μg/ml rhIFN-γ in Tris Buffer Salt (TBS) dropped on the membrane (strips). After adsorption of antigenic solution, the membranes transferred into 2% BSA in TBS for 1h and incubated with TBS-T (0.02M Tris, 0.15M NaCl, 0.05% Tween20 pH7.5) diluted sera for 1h. Then treated with a 1/1000 dilution of enzyme conjugated antibody (HRP) and finally the Diaminobenzamidine (DAB) solution used as substrate along with 0.1% H_2O_2. After appearing of the dots, the reaction stopped by H_2O and dried at air.

Sandwich ELISA technique. In this technique, polyclonal antibodies from rabbit and mouse were used as the first and second antibodies respectively. At first, antibody concentration was setting up for using in sandwich ELISA system by serial dilution of antibodies from rabbit and mouse. A concentration of 170μg/ml of rabbit antibody was coated in wells of ELISA plate and kept at 4°C overnight. Then the 2% BSA in TBS-T used for blocking of the wells for 1h. A serial concentration of

rhIFN-γ (from 10μg/ml to 0.01ng/ml) were added onto the wells and incubated for 3-4h in room temperature. In the next step, after 1h the mouse polyclonal antibody was added to the wells in a 1/300 dilution as second antibody. The anti-mouse conjugated antibody was used and incubated at room temperature for 1h. Finally the *O*-phenylenediamine (OPD) and H_2O_2 mixture were added to the wells and the color reaction stopped after 30min and the adsorption was read in 492nm.

Results and Disscution

After precipitation of the rabbit polyclonal antibody with 50% ammonium sulfate, and based on the adsorption at 230, 260 and 280nm the protein concentration was calculated 37mg/ml.

In consequence, the titer of rabbit (1/20000) and mouse (1/10000) polyclonal antibody was confirmed by dot blot technique. For Sandwich ELISA technique, an optimization step for three variants including first antibody, antigen and second antibody was performed. Based on the results, the concentration of 170μg/ml for rabbit polyclonal antibody precipitated with ammonium sulfate and 1/300 dilution of mouse serum were determined.

According to the diagram 1, the sensitivity of this system decreases in concentration upper than 1250ng/ml rhIFN-γ and the slope of diagram reachs to zero. It seems this designed system can detect amount between 40-1250ng/ml IFN-γ efficiently (Diagram 2).

In recent years, there is a great progress in knowledge of the biology of cytokins and their physiological, immunological and clinical effects for treatment of infectious diseases and cancers (Bharat & Raj 1995). For this designing a rapid and sensitive enzyme-immunoassay for native and recombinant human interferon gamma is important. In other reports, designing of such system have been performed for IFN-γ and since the second antibody has been monoclonal antibody in conjugated form with HPR (Gallali *et al* 1987, Jitsukawa *et al* 1987, Cuury *et al*

1987, Oda *et al* 1986). The minimum concentration of IFN-γ, which could be detected, is as low as 0.3ng/ml (Gallali *et al* 1987).

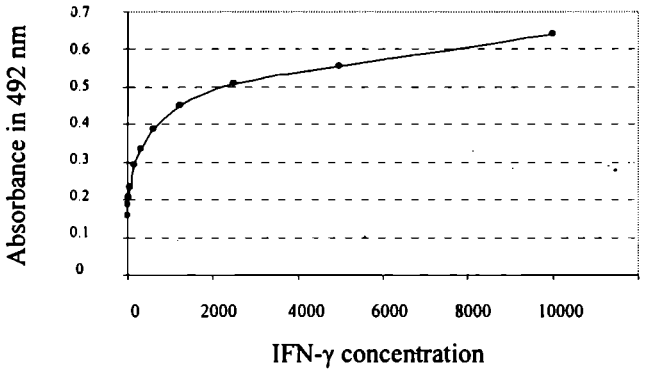

Diagram 1. *IFN-γ concentretion curve*

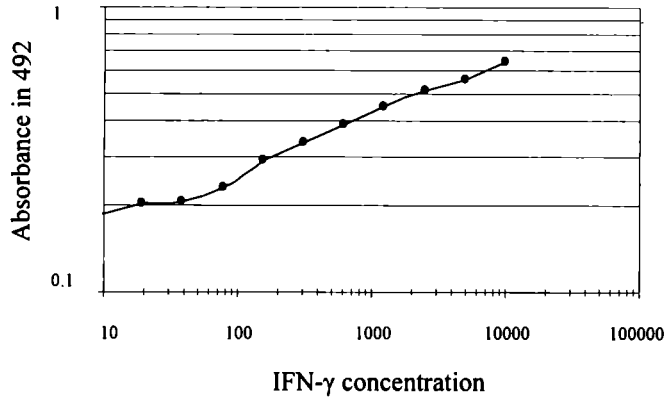

Diagram 2. *Exponential curve for absorbance of different concentration of IFN-γ in ELISA system*

In this study we have designed a sandwich ELISA system to detect the least amount of IFN-γ using rabbit and mouse polyclonal antibodies. In addition to determining the concentration and amount (native and functional) of IFN-γ according to its binding to active sites of polyclonal and monoclonal antibodies this method can be used to study recombinant IFN-γ. A minimum concentration of

40ng/ml of IFN-γ can be detected by this method. As IFN-γ concentration in plasma is too low (in rang of pg/ml) the use of a monoclonal antibody and a purified antibody could be more appropriate for this system. Therefore, this technique can be used for determination of the high level of IFN-γ in the serum of patient with granulum (CGD) before and after IFN-γ injection. Finally cross reactivity of hIFN-γ with other cytokines in this method and purified rhIFN-γ refolding need future studies.

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