JOURANL OF IMMUNOASSAY, 14(1&2), 83-97 (1993)

MEASUREMENT AND COMPUTATION OF MURINE INTERLEUKIN-4 AND INTERFERON-γ BY EXPLOITING THE UNIQUE ABILITIES OF THESE LYMPHOKINES TO INDUCE THE SECRETION OF IgG1 AND IgG2a

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ABSTRACT

A specific and new method for measuring Interleukin-4 and Interferon-γ, based on the estimation of IgG1 and IgG2a isotypes secretion from B cells is described. An antagonizing effect of Interferon-γ in the production of IgG1 induced by Interleukin-4 was neutralized by using antibody to Interferon-γ. Similarly, the interference of Interleukin-4 in the Interferon-γ mediated enhancement of IgG2a production was blocked by anti-Interleukin-4 antibody. The high concentrations of Interleukin-4 and Interferon γ inhibited the secretion of IgG1 and IgG2a respectively. Therefore, in the assay described, the samples containing the cytokines were so diluted that their activity fell into the non-inhibitory zone. A computer program has also been developed for determining the concentrations of lymphokines.

Keywords: Interleukin-4; Interferon-γ; Computer Program; Immunoglobulin Isotype

Abbreviations : rIL-4, recombinant interleukin-4; rIFN-γ, recombinant interferon-γ; LPS, lipopolysaccharide; HRP, horse radish peroxidase; OPD, orthophenylene diamine.

INTRODUCTION

T-helper cells have been divided into T_H-1 and T_H-2 subsets on the basis of their lymphokines profile. T_H-1 cells secrete IL2, IFN-γ, lymphotoxin, whereas T_H-2 cells secrete IL-4, IL-5, IL-6. However both type of these T_H cells secrete IL-3, GM-CSF and TNF-α (1). Interleukin-4 is known to affect the growth and differentiation of

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immunologically competent cells, such as B cells, T cells and cells of myeloid lineage (2,3). Interferon-γ is a pleiotropic lymphokine and acts on a number of cell types viz., macrophages, B cells, cytotoxic T cells and NK cells (4). Interleukin-4 promotes the switching of IgG1 and IgE(5). Interferon-γ markedly enhances IgG2a secretion from LPS activated B cells and it is also reported that IL-4 and IFN-γ reciprocally regulate the functions of each other (6). These lymhokines have several other biological activities which regulate the immune responses and in turn play an important role in the development of protective immunity against certain allergic and parasitic infections (7,8) . Thus, IL-4 and IFN-γ may have potential clinical and therapeutical applications as an immunomodulatory compounds.

During the past decade, several methods have been developed for the estimation of IL-4 and IFN-γ. These assays are based on the a) induction of proliferation or inhibition of the growth of various cell lines (9,10), b) induction of certain surface molecules like Ia, CD23 etc. (11,12) and c) enzyme-linked immunosorbent assay (ELISA) (13) . However, these methods have certain technical difficulties as i) the maintenance of the cell lines is a cumbersome process and requires a continuous supply of the expensive growth factors; ii) in addition to the lymphokines to be tested, assay based on proliferation of cell lines, generally respond to more than one cytokine, and iii) given cell line maintained in vitro, expressing a particular receptor, may mutate to be less responsive. The drawback of the ELISA is that it does not necessarily demonstrate the biologically active status of the lymphokines.

To overcome some of these problems, we have developed a reliable, sensitive and reproducible assay for the estimation of IL-4 and IFN-γ, on the basis of their unique characteristics to induce IgG1 and IgG2a secretions respectively, from LPS activated B

cells. The simplicity and accuracy have been further increased by developing a computer program for data analysis.

MATERIALS AND METHODS

Animals: Inbred female BALB/c mice were received from National Institute of Nutrition, Hyderabad, India.

Antibodies, Reagents and Lymphokines: The anti-Thy1.2 antibody was purchased from Cedarlane (Ontario, Canada). Monoclonal antibodies specific for murine IL-4 (11B11) and IFN-γ (R46A-2) were obtained from Texstar (Texas,USA) and Holland Biotechnology (Leiden, Netherlands) respectively. Rabbit anti-mouse IgG, orthophenylenediamine and LPS were procured from Sigma (St. Louis, USA). Goat-anti-mouse IgG1-biotin, goat anti-mouse IgG2a-biotin and streptavidin-HRP were obtained from Sera-Lab (Sussex, England). Percoll was purchased from Pharmacia (Piscataway, N.J.). Recombinant mIFN-γ was purchased from Holland Biotechnology and rmIL-4 was supplied by Genzyme (Bostan, USA). RPMI-1640 (GIBCO) supplemented with 10% FCS (Sera Lab), L-glutamine, (2mM), 2ME (0.05mM), penicillin (50μg/ml) and streptomycin (50 μg/ml) was used in all the cultures.

Preparation of resting B cells: Enriched population of resting B cells were obtained from spleen cells suspension from which T cells were depleted by the treatment with anti-Thy1.2 antibody and baby rabbit complement. Small resting B cells were isolated on percoll density gradients by a modified method (14) . Briefly, gradients consisting of 100%, 70% and 50% percoll were prepared. T cells depleted spleen cells were layered on the top of the 50% percoll gradient and centrifuged at 1600 x g. The high density small cells were collected from the 70-90% percoll interface.

Functional assay: Small resting B cells (1×10^4) were cultured in triplcate wells in 96-well plates (Costar, Cambridge, NA) in 200μl RPMI-1640 medium containing 10 % FCS and 20 μg/ml LPS at 37°C in a 7% CO_2 atmosphere. Various concentrations of lymphokines were added simultaneously with or without antibodies. On day 6, the cell supernatants were collected and IgG1 and IgG2a levels were estimated by the standard ELISA procedure (15). Briefly, the duplicate wells of the microtitre ELISA plates (Costar) were coated overnight with rabbit anti-m IgG (5μg/ml) at 4°C. The unbound sites were blocked with 2% BSA and then standard mIgG1 and mIgG2a and serial log 10 dilutions of culture supernatants were added for 2 hrs. at 37°C. Isotypes IgG1 and IgG2a were detected using biotinylated goat antimouse IgG1 and IgG2a antibodies respectively. After incubating the plates at 37°C for 2hrs. streptavidin - HRP was added. The usual steps of washing using PBS-Tween20 was carried at each step. Color developed due to the substrate OPD was visualized at 492nm.

Data Analysis and Development of Computer Program

A). Analysis of Standard data: The values of IgG1 and IgG2a were fed to microcomputer by using keyboard. The program exploited the IgG1 and IgG2a data information of known standards and derived a linear interpolation formula for determining the concentration of IL-4 and IFN-γ.

The values of IgG1 and IgG2a (dependent variable) and concentration of lymphokines (independent variable) of known standards were utilized for calculating linear interpolation formula. The initial linear portion of the curve with positive slope was depicted as a usable range. This was selected by using a tunable algorithm. The algorithm at first calculated the slope of all consecutive points of the curve and simultaneously checked the slope at the different points from lower to higher concentrations, and se-

lected the points till the slope became negative. The portion of the curve beyond the usable range was considered as the zone of inhibition. The data of usable range of the curve was fitted by using following linear equation

$$I = A_0 + A_1 \times C \tag{1}$$

Where I is the amount of IgG1 secreted from the B cell and C is the concentration of Interleukin-4 present in the culture well. A_0 and A_1 represent the constant and slope of the curve respectively. The values of A_0 and A_1 were calculated by fitting the data in the usable range using least square curve fitting method. The equation (1) can therefore be written as

$$C = (1/A_1) \times I - (A_0/A_1) \tag{2}$$

The equation (2) represents the linear interpolation formula for calculating the concentration of IL-4. In a similar way, the equation for determining the IFN-γ concentration was also derived. The standard curves for rIL-4 and rIFN-γ are shown in Fig. 1b & 2b.

B). Estimation of IL-4 and IFN-γ concentrations The lymphokines concentrations in the samples were calculated from the values of IgG1 and IgG2a using equation (2). It may be mentioned that this formula is applicable only when the concentration of lymphokines is in the usable range. One of the critical problems in measuring the high concentrations of IL-4 and IFN-γ is that beyond an optimum concentration, these lymphokines downregulate the secretion of the isotypes motivated by them. To overcome this problem, the samples were diluted so that at least the activity of one of its dilutions should fall into the usable range. The concentration of lymphokines at the highest dilution was calculated from equation (2). The value obtained was then multi-

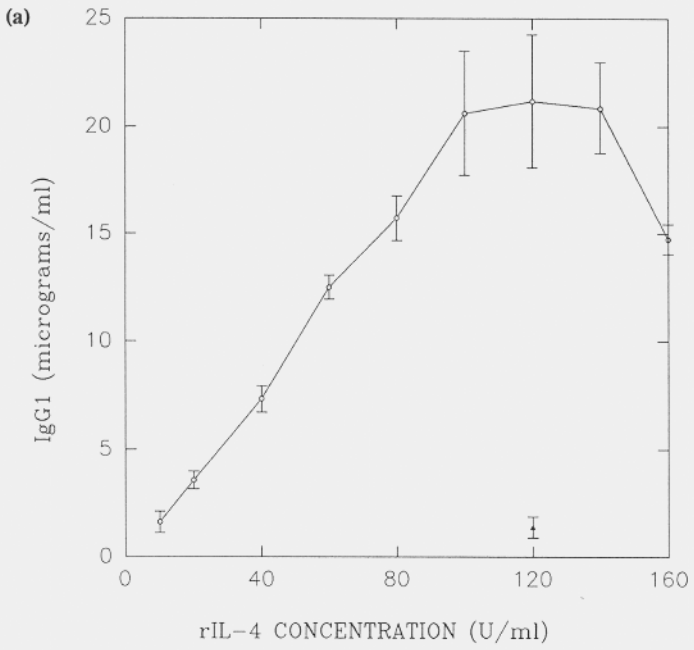

Fig. 1. (a) Recombinant IL-4 mediated induction of IgG1 secretion by LPS activated resting B cells. The cells ($1x10^4$) were cultured with LPS (20μg/ml) and rIL-4 in triplicate wells in the presence (▲) or absence (-O-) of anti-IL-4 antibody (10μg/ml) and the secretion of IgG1 was measured by ELISA as described in materials & methods. (b) Standard curve was fitted by least square curve fitting method using the usable range of the curve of Fig. 1. (a). In the absence of rIL-4, the background value for LPS alone was 60 ± 9ng/ml culture. The data represents the mean ± SD of the three experiments.

concentrations of earlier dilutions were also measured from the above equation till the concentrations fell in usable range. Finally, the mean of all dilutions in the usable range was calculated which represented the true concentrations of lymphokines in the samples. While calculating the concentrations of the lymphokines, the background value of IgG secreted by B cells stimulated with LPS alone was subtracted. The computer program takes care of all the above calculations.

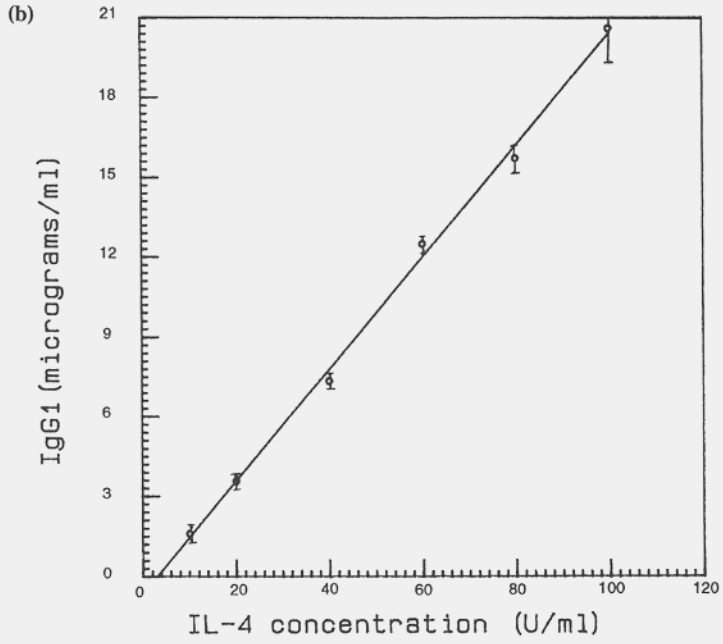

Fig. 1. Continued

RESULTS

Interleukin-4 and IFN-γ selectively promote LPS activated B cells to secrete IgG1 and IgG2a respectively. It is well known that IL-4 and IFN-γ elevate the secretion of IgG1 and IgG2a at certain optimum concentration only and at higher concentration these lymphokines inhibit the production of the above isotypes. Accordingly, we first decided to titrate the optimum concentration of these lymphokines for the differentiation of B cells. Purified murine resting B cells were stimulated with LPS and increasing concentrations of rIL-4 and rIFN-γ (Fig. 1a & 2a). IgG1 (21.60μg/ml) levels peak at rIL-4 concentration of 120U/ml as compared to the control (0.6 μg/ml) (Fig. 1b). Further, increase in rIL-4 concentration (320U/ml) resulted in progressive decline of IgG1

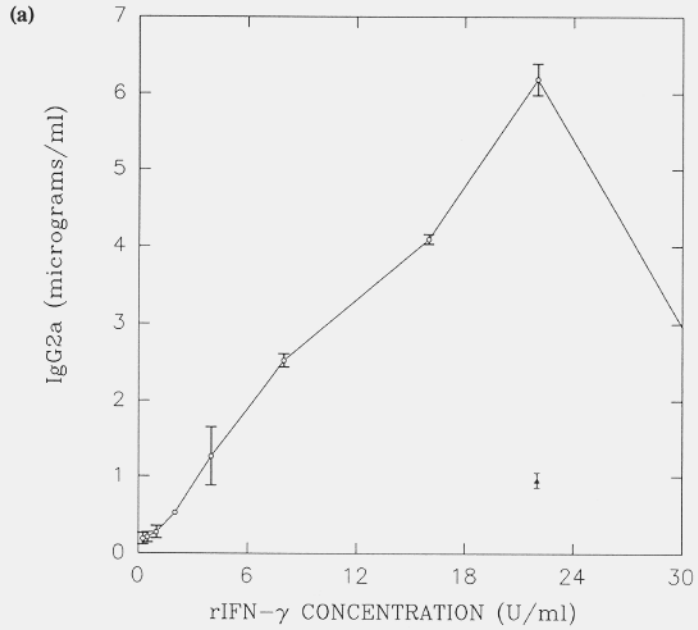

Fig. 2. (a) Recombinant IFN-γ mediated induction of IgG2a secreation by LPS activated resting B cells. The cells ($1x10^4$) were cultured with LPS (20μg/ml) and rIFN-γ in triplicate wells in the presence (▲) or absence (-O-) of anti-IFN-γ antibody (4 μg/ml) and the secretion of IgG2a was measured by ELISA as described in materials & methods. (b) Standard curve was fitted by least square curve fitting method using the usable range of the curve of Fig. 2. (a). In the absence of rIFN-γ, the background value for LPS alone was 44 $\pm$ 5ng/ml culture. The results are the mean values $\pm$ SD of three experiments.

(12.25μg/ml) (Fig. 1a). Interferon-γ caused a striking increase in IgG2a secretion. Incorporation of rIFN-γ at a concentration of 22U/ml resulted in the maximum secretion of 6.63μg/ml of IgG2a (Fig.2 b). Further, addition of rIFN-γ to the culture wells inhibited the secretion of IgG2a (Fig. 2a).

In order to test the specificity of the response measured for rIL-4 and rIFN-γ, monoclonal antibodies against these lymphokines were incorporated into certain culture wells. These antibodies significantly suppressed IL-4 mediated IgG1 secretion (21.60 μg/ml to 1.34 μg/ml) and IFN-γ mediated IgG2a secretion (6.63 to 0.98 μg/ml) (Fig.1a

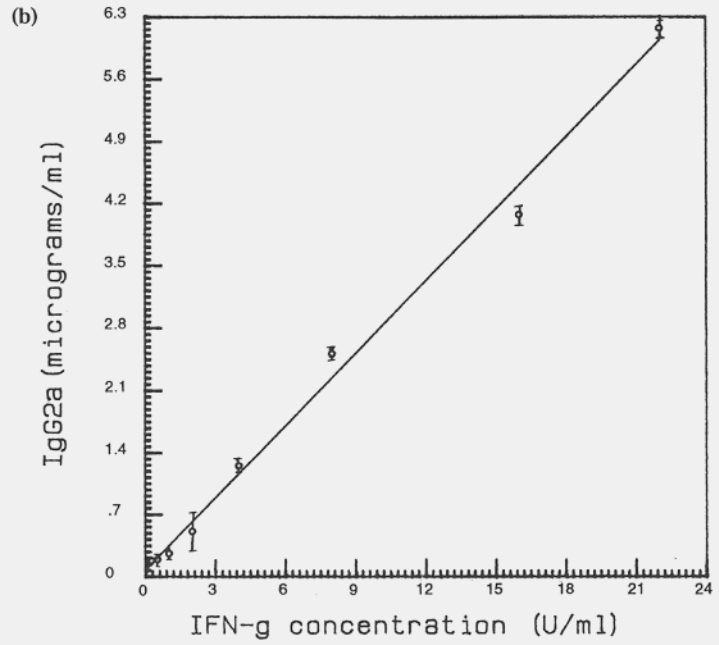

Fig. 2. Continued

& 2a). Antibodies against IFN-γ (4 μg/ml) and IL-4 (10μg/ml) had no effect on the concentration of IgG isotypes secreted by B cells treated with LPS only.

Measurement of IL-4 in the presence of IFN-γ and vice-versa

Interleukin-4 and IFN-γ have reciprocal regulatory effects on each other. Therefore, it is difficult to precisely measure the activity of one of the lymphokines in the presence of the other. Interleukin-4 alone induced 7.86, 10.61 and 19.34μg/ml of IgG1 at the concentrations of 40, 60 and 100U/ml respectively. As expected, there was significant decrease in the IL-4 enhanced production of IgG1 isotype when the cells were cultured in the presence of 100U/ml and 10U/ml of rIFN-γ. Addition of 4μg/ml of anti-IFN-γ

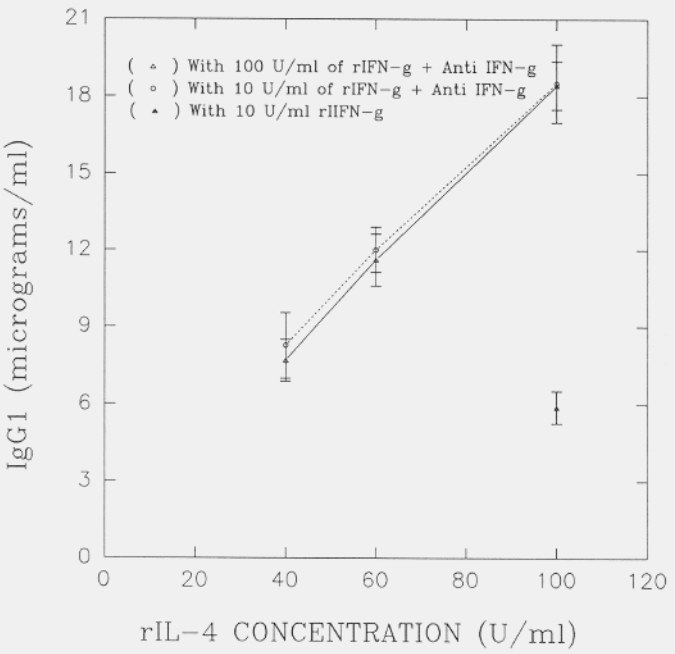

Fig. 3. Blocking of the inhibititory activity of rIFN-γ in rIL-4 mediated enhancement of IgG1 production. Resting B cells ($1x10^4$) were stimulated with LPS (20 μg/ml) and cultured in triplicate wells in the presence of various conc. (40,60 and 100 U/ml) of rIL-4. In certain wells rIFN-γ (10 and 100 U/ml) was added and its inhibitory activity was neutralized with 4 μg/ml of anti-IFN-γ antibody. The IgG1 level was estimated as described in Fig. 1a. Mean of triplicate assays are shown.

antibody abrogated the interference created by IFN-γ. It may be noted that IgG1 level remained unaffected in the aforementioned conditions (Fig. 3).

The experiments were also standardized to monitor the activity of IFN-γ in the presence of IL-4. Recombinant Interleukin-4 significantly lowered the level of IgG2a secretion induced by rIFN-γ (Fig 4). Supplementation of the cultures with anti-IL-4 antibody (10μg/ml) eliminated the obstructing effect of 100U/ml and 600U/ml of rIL-4 on the activity of rIFN-γ . The cells were cultured with 5, 15 and 20U/ml of rIFN-γ

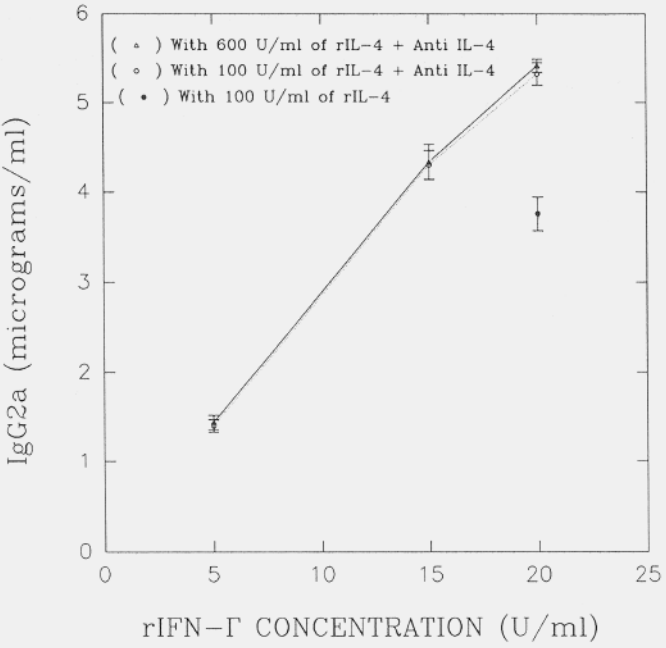

Fig. 4. Blocking of the inhibititory activity of rIL-4 in rIFN-γ production mediated enhancement of IgG1 production. Resting B cells ($1x10^4$) were stimulated with LPS (20 μg/ml) and cultured in triplicate wells in the presence of various conc. (5,15 and 20 U/ml) of rIFN-γ. In certain wells rIL-4 (100 and 600 U/ml) was added and its inhibitory activity was neutralized with 10 μg/ml of anti-IL-4 antibody. The IgG2a level was estimated as described in Fig. 1a. The results are expressed as mean of the triplicate experiments.

along with 100U/ml and 600U/ml of rIL-4 in the presence of antibody to IL-4. The levels of IgG2a secretion (1.44,4.33 and 5.41 μg/ml) under these conditions were comparable when no antibody was added into the culture (Fig. 4).

Accuracy and sensitivity of the assay

In order to determine the accuracy and reproducibility of the present bioassay, reference samples containing various quantities of rIL-4 and rIFN-γ ranging from low to very high conc. (20 U/ml, 600 U/ml & 1000 U/ml of rIFN-γ and 100 U/ml, 600 U/ml &

Table. 1. Sensitivity of the present method for the estimation of lymphokines .

Resting B cells ($1x10^4$) were cultured with LPS (20μg/ml) and specified units of rIL-4 and rIFN-γ as described earlier. On day 6, the supernatants from triplicate wells were collected and analysed for calculating the concentrations of IL-4 and IFN-γ. The results are the mean values $\pm$ SD of three experiments.

INTERLEUKIN-4(U/ml)			INTERFERON-γ(U/ml)		
Actual conc.*	Estimated conc. (Mean±SD)	%error	Actual conc.*	Estimated conc. (Mean±SD)	%error
10000	10132± 337.27	1.32	1000	1016± 63.59	1.6
600	615.7± 25.40	2.61	600	584.3± 30.66	2.61
100	100.8± 2.03	0.80	20	19.57± 1.00	2.15

* As defined by the suppliers.

10000 U/ml of rIL-4) were prepared and tested for there ability to induce the secretion of IgG1 and IgG2a by LPS activated B cells respectively. The activity obtained for IL-4 by employing our assay system is comparable to the concentrations of rIL-4 and rIFN-γ specified by the suppliers. The percentage of error for IL-4 ranges from 0.80 to 1.32 and for IFN- γ 1.6 to 2.15. It may be reiterated that the percentage of error remained insignificant (1.32) even when estimating 10000 U/ml of rIL-4 and 1.6 for 1000 U/ml of rIFN-γ (Table 1).

Our method is shown to be quite reproducible and sensitive. One can measure IL-4 and IFN-γ as low as 5U/ml and 1U/ml respectively which is comparable with other methods. Siegel *et al.*, (21) have demonstrated that with the use of Ramos G6.C10 cell lines, sensitivity for human IL-4 was 3U/ml. While using T cell lines, it is reported that the sensitivity limit for murine IL-4 is 3-10U/ml and for murine IFN-γ is 200pg/ml (22).

Our method is highly accurate for measuring low as well as very high concentrations of IL-4 and IFN-γ. The level of error is negligible (Table 1).

DISCUSSION

The available bioassays for human and murine IL-4 or IFN-γ are based upon proliferation or inhibition of the growth and expression of Ia, CD23 etc., of certain cells. For this purpose, IL-4 and IFN-γ responsive cell lines like HT-2, CTLL-2, Ramos G6.C10 and WEHI-279 have extensively been used (11-13) The methods employing cell lines have certain drawbacks as T-cell lines that proliferate in response to IL-4 also synergize with other lymphokines (16,17). Moreover, IL-4 induced expression of Ia and CD23, and IFN-γ elicited secretion of IgG2a by B cells are downregulated by IFN-γ and IL-4 respectively (18,19,6). Surprisingly, no extensive efforts have been made to rule out precisely the possible interference of these lymphokines in the bioactivity of each other. In order to address these issues, in the present manuscript, we have exploited the unique feature of these lymphokines to promote the secretion of IgG1 and IgG2a by LPS activated B cells (20).

Interleukin-4 and IFN-γ have been shown to antagonize the biological activity of one another (6). Therefore, it would be difficult to simultaneously measure these cytokines. To ensure this, we have neutralized the activity of IFN-γ while measuring IL-4 by addition of anti-IFNγ antibody, and similarly the possible counteraction of IL-4 in the activity of IFN-γ was blocked by anti-IL-4 antibody. This has been clearly demonstrated in Fig. 3 and 4. It may be recalled that the levels of IgG1 remained the same when LPS stimulated B cells were differentiated by IL-4, or IL-4 and IFN-γ in the presence of anti-IFN-γ antibody. Likewise, IFN-γ mediated IgG2a levels also remained the same irrespective of cells cultured with IFN-γ, or IFN-γ and IL-4 in the presence of

anti-IL-4 antibody. Thus, the method described here can be of an additional advantage for the estimation of functional status of these lymphokines.

The program, for analysis and calculation of the data is written in GW-BASIC and can be run on a IBM compatible PC under MS-DOS version 2.0 or higher, and requires a CGA or EGA or VGA card. It runs without a math co-process and a hard copy can be plotted on HP-GL plotters or on the printer by pressing the Print Screen key. The program is simple to use. This is a menu driven computer program and provides data storage facility on a Disk. A copy of the source and executive version of program can be obtained by sending a 5.25" floppy disk to the author.

Acknowledgments

This is a communication no. 006/92 from Institute of Microbial Technology, Chandigarh, India. We thank Mr. R. Das for technical assistance and Mrs. S. Batra for secretarial assistance. We also thank Drs. C.M. Gupta and V. Das for reviewing the manuscript.

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