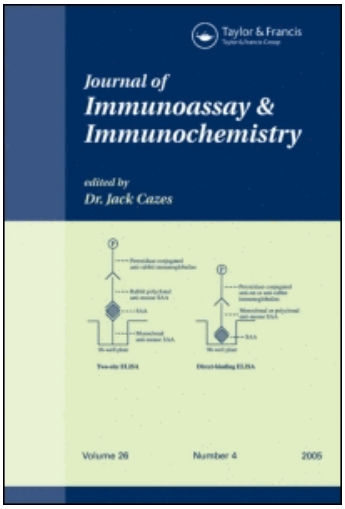

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A Simple, Sensitive, Dual mAb Based Elisa for Murine Gamma Interferon Determination: Comparison with two Common Bioassays

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A SIMPLE, SENSITIVE, DUAL mAb BASED ELISA FOR MURINE GAMMA INTERFERON DETERMINATION: COMPARISON WITH TWO COMMON BIOASSAYS

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ABSTRACT

Three assays of murine IFNγ are compared in terms of sensitivity, intra- and inter-assay variability, specificity and simplicity. The widely used viral inhibition assay requires 48 hours, necessitates continuous maintenance and optimization of fibroblast growth, and exhibits the lowest sensitivity. Inhibition of WEHI-279 B cell [^{3}H]thymidine incorporation requires 48-60 hours to quantitate IFNγ production, can be subject to non-specific inhibition, and is also labor intensive. In both bioassays, specificity must be determined by the analysis of duplicate samples in the presence of neutralizing, IFNγ-specific mAb. In contrast, a 24 hour, dual mAb ELISA, in which IFNγ is captured by immobilized, purified rat IgG_1 XMG 1.2 mAb and identified with biotinylated mAb R4-6A2 and streptavidin-alkaline phosphatase detects IFNγ production >0.05 U/ml. The quantitative range in this assay is typically from 1-100 U/ml. In addition to providing the greatest specificity and shortest duration, this ELISA exhibits the lowest coefficient of variation of the three assays compared. Collectively, assay characteristics such as sensitivity, absence of interference by other proteins, reproducibility, speed and simplicity support the conclusion that this dual mAb based sandwich ELISA represents a substantial improvement over inhibition of viral cytopathic effect or inhibition of WEHI-279 bioassays for characterization of antigen- or mitogen-driven IFNγ production.

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INTRODUCTION

Gamma interferon (IFNγ)[1], an inducible cytokine produced by T and NK cells following antigen-specific or polyclonal activation, was initially characterized on the basis of its anti-viral properties and is now recognized to be highly pleiotropic in function. Much recent attention has been given to its diverse immunoregulatory properties, in particular its role in induction and regulation of IgE responses (1-4).

Characterization of IFNγ production requires assays with very high sensitivity as the level of cytokine production elicited following antigen specific stimulation of normal T cells in short term primary cultures is typically two or more orders of magnitude below that observed following activation of long term T cell clones or hybridomas. A variety of bioassays have been used to quantify IFNγ production in tissue culture supernatants derived from murine and human primary cultures, including viral inhibition assays (5) and inhibition of WEHI-279 cell proliferation (6), MHC class II induction (6,7), and others. Most of these assays offer a high degree of sensitivity (as low as 1-2 U/ml in experienced hands). However, they share the disadvantage of all bioassays that other cytokines present in the sample may synergize with or antagonize the effects of the analyte on the bioassay's target cell. Moreover, particularly in the case of inhibition assays, non-specific inhibitory effects from metabolites present in exhausted tissue culture supernatants can be problematic. Consequently, the extent of this interference, and the specificity of the results obtained, must be determined for each sample by comparison of replicates

cultured in the presence and absence of neutralizing antibodies to the cytokine in question.

An approach to circumvent the inherent disadvantages of bioassays lies in cytokine specific immunoassays. Although a number have been established using one monoclonal antibody paired with a polyclonal xenogeneic antiserum, this approach suffers from the inherent limitations involved in generation of the polyclonal component. Here, we describe a simple and highly sensitive ELISA for murine IFNγ using two mAb that are readily available. The sensitivity of this ELISA is compared with two of the most widely used bioassays of murine IFNγ. The ELISA was found to offer practical and quantitative advantages and to perform well when measuring physiological concentrations of IFNγ.

MATERIALS AND METHODS

Cytokines

Purified murine rIFNγ (Holland Biotechnology, Leiden, Netherlands) derived from Chinese Hamster Ovary cells, and as a source of natural IFNγ, Concanavalin A stimulated mouse spleen cell culture supernatants, calibrated against WHO-NIAID international reference reagent Gg02-901-533 (provided by Dr. C. Laughlin, NIAID, Bethesda, MD) were used in each assay. Murine IFNα/β and human rIFNγ were provided by Dr. F. Jay, (Univ. of Manitoba, Winnipeg, MB), murine rIL-4 was provided by Dr. W. Paul, (NIAID, Bethesda, MD) and murine rIL-2 was purchased from Genzyme, Boston, MA).

Inhibition of Viral Cytopathic Effect Bioassay

A mycoplasma free subline of mouse L-929 fibroblasts, designated LB, were provided by Dr. F. Jay (Univ. of Manitoba). Actively proliferating cultures were trypsinized (0.05% Trypsin-EDTA, Gibco-BRL, Burlington, ON), resuspended as a single cell suspension in complete medium (8) at 4 x 10^4/ml and plated in 96 well flat bottom plates at 100 ul/well then cultured for 3 hours at 37° to re-establish an adherent monolayer. At this time, recombinant and natural IFNγ standards were added as 100 ul samples of two fold dilutions in complete medium. Following 18 h. additional incubation at 37°, the capacity of IFNγ to protect LB cells from viral cytopathic effect was determined by addition of 100 ul of diluted encephalomyocarditis virus, in complete medium, to all except unchallenged control wells. Virus infected fibroblasts cultured in IFNγ deficient, complete medium were used as a negative control. Virus was used at the predetermined minimum concentration necessary to elicit 100% cytopathic effect in unprotected, actively proliferating fibroblast cultures. The plates were cultured for a final 24-30 h. at 37° after which multiwell plates were emptied by rapidly flicking out the supernatant and blotting them firmly on paper toweling. Approximately 100 ul crystal violet solution (0.25% crystal violet, 0.9% NaCl, 20 mM Tris HCl, 20% methanol v/v, pH 7.5) was added to each well and, after 10-30 minutes at room temperature, the plates were gently rinsed with running tap water.

Specificity in this bioassay was determined by concurrent analysis of replicates in the presence of purified anti-IFNγ mAb XMG 1.2 (9) (hybridoma provided by Dr. T. Mosmann, Univ. of Alberta) at

concentrations sufficient to block the activity of >200 U/ml IFNγ. Supernatant activity was attributed to IFNγ if ≥90% of protection from viral cytopathic effect was abolished by the inclusion of this anti-IFNγ mAb. The concentration of biologically active IFNγ was expressed as a reciprocal titre based on the sample dilution at which 50% inhibition of viral cytopathic effect was observed.

WEHI-279 Bioassay

Independent samples of this B lymphoma cell line were provided by Dr. M. Hagen, (Univ. of Iowa, Iowa City, IA) and Dr. A. Kelso (Walter and Eliza Hall Inst, Melbourne, Australia). IFNγ concentrations were determined on the basis of its ability to inhibit WEHI-279 proliferation essentially as described (6), but using an abbreviated assay. In brief, WEHI-279 cells in log phase growth (viability >99%) were seeded at 10^4 cells/100 ul culture medium (8) in each well of 96 well flat-bottom plates (Corning Science Products, Corning, NY). 100 ul of two fold dilutions of IFNγ containing culture supernatants or rIFNγ in complete medium were added and the plate was incubated at 5% CO_2, 37°C for 48 h. Cells cultured in IFNγ deficient medium were used as a negative control. Tritiated thymidine (0.5 uCi per well) was added for an additional 6-8 hours of culture and ^{3}H-TdR incorporation was determined by liquid scintillation counting. Specificity in this bioassay was determined by concurrent analysis of replicates in the presence of purified anti-IFNγ mAb XMG 1.2 at concentrations sufficient to block the activity of >200 U/ml IFNγ. Inhibition of WEHI-279 growth was attributed to IFNγ if ≥90% of growth inhibition

was abolished by the inclusion of anti-IFNγ mAb. Data are presented as mean dpm ± SEM.

IFNγ ELISA

Preparation and Purification of Anti-IFNγ mAb

Hybridomas XMG 1.2, (Rat IgG_1, provided by Dr. T. Mosmann, Univ. of Alberta, Edmonton, AB; available as purified mAb from PharMingen, San Diego, CA) and R4.6A2, (Rat IgG_1, obtained from the American Type Culture Collection, Rockville, MD) were grown to exhaustion in complete medium.

Supernatants were purified by ion exchange chromatography then gel filtration using the Pharmacia Biopilot system (Pharmacia Fine Chemicals, Piscataway, NJ) (10). Briefly, culture supernatants (0.5 to 3.5 *l*) were extensively dialysed against low ionic strength citrate buffer (0.01 M, pH 5.3), then concentrated via ion exchange chromatography using S-Sepharose 35/100. The mAb containing fraction was eluted using a gradient of increasing ionic strength (NaCl 0-0.8 M, Na citrate 0.01 M, pH 5.3) then applied directly to a gel filtration column (Superdex 200, 60/600 in PBS) with the mAb collected in that fraction corresponding to 150-200 kD. The identity and purity of the mAb were determined by ELISA. The final purity of the rat mAbs obtained by this approach was typically >85% as determined by comparison of total protein present in the purified material as determined by BCA (Pierce, Rockford, IL) protein assay compared with the concentration of total rat IgG as determined by ELISA.

Biotinylation of R4.6A2

Purified R4.6A2 was dialized against $NaHCO_3$ (0.1 M, pH 8.15) and concentrated to 1-2 mg/ml using a Centriprep-30 concentrator (Amicon, Oakville, ON). A biotin-spacer conjugate (BNHS; D-Biotin-e-aminocaproic acid N-hydroxysuccinimide ester, Boehringer Mannheim, Montreal, PQ) was dissolved in anhydrous DMSO (Fisher Scientific, Ottawa, ON) at 2 mg/ml and added to the antibody at the ratio of 1:4 (antibody:BNHS, v/v). The reaction was allowed to proceed for 2 hours at room temperature in the dark, following which unbound biotin was removed by extensive dialysis against PBS at 4°C. This reagent was stable for at least 8 months at 4°C in NaN_3 (0.01%).

ELISA procedure

96 well ELISA plates (Corning 25805, Corning Science Products) were coated with 100 ul/well of XMG 1.2 at 5-10 ug/ml in coating buffer (0.05 M $NaHCO_3$ buffer, pH 9.6). Plates were kept at 4°C overnight, or 37°C for 2 hours, then emptied, blocked (0.2 ml/well of 1% BSA in PBS, pH 7.4) for 2h. at room temperature, and extensively washed (0.05% Tween 20 in PBS). IFNγ standards and samples were prepared in dilution buffer (0.5% Tween 20, 0.5% BSA in PBS) and added at 0.1ml/well. Following 3 h incubation at 37°C and subsequent washing, biotinylated R4.6A2 was added for an overnight incubation at 4°C. The next day, the plates were washed, streptavidin-alkaline phosphatase (Jackson Immunoresearch, West Grove, PA) was added (0.1 ml/well, 1 h. 37°C) at the recommended dilution, the plates were washed again and 100 ul/well of p-nitrophenyl phosphate (Sigma Chemical Co.) in 0.5mM $MgCl_2$, 10%

diethanolamine, pH 9.8) was added. The plates were read by automatic ELISA reader at 30, 60, 90, and 120 min.

Statistics

Inter-assay variability was determined using a minimum of 4 assays for natural and rIFNγ at 6 concentrations ranging from 60 U/ml to <2 U/ml. Intra-assay variation was determined with a minimum of 5 replicates per plate per sample per concentration. The data are evaluated by coefficient of variation analysis.

RESULTS

Inhibition of viral cytopathic effect assay

The capacity of IFNγ containing, tissue culture supernatants to protect mycoplasma free L-929 cells from lysis by virus was evaluated (Figure 1). This widely used assay offers reasonable sensitivity, 2-4 U/ml IFNγ under optimal conditions. However, its utility is restricted by a several considerations. It exhibits a lack of specificity (Figure 1), requiring that all samples be tested in the presence and absence of neutralizing anti-IFNγ antibodies. The method is somewhat cumbersome in that there is a need to optomize the concentrations of fibroblasts used and virus added for maximal sensitivity. Moreover, fibroblasts need to be continuously maintained in culture, with the sensitivity of the assay dependent on the fibroblasts being in log phase growth. Comparison of data obtained in different laboratories is complicated by the susceptibility of the endpoint to variations in the cell strains and virus stocks used.

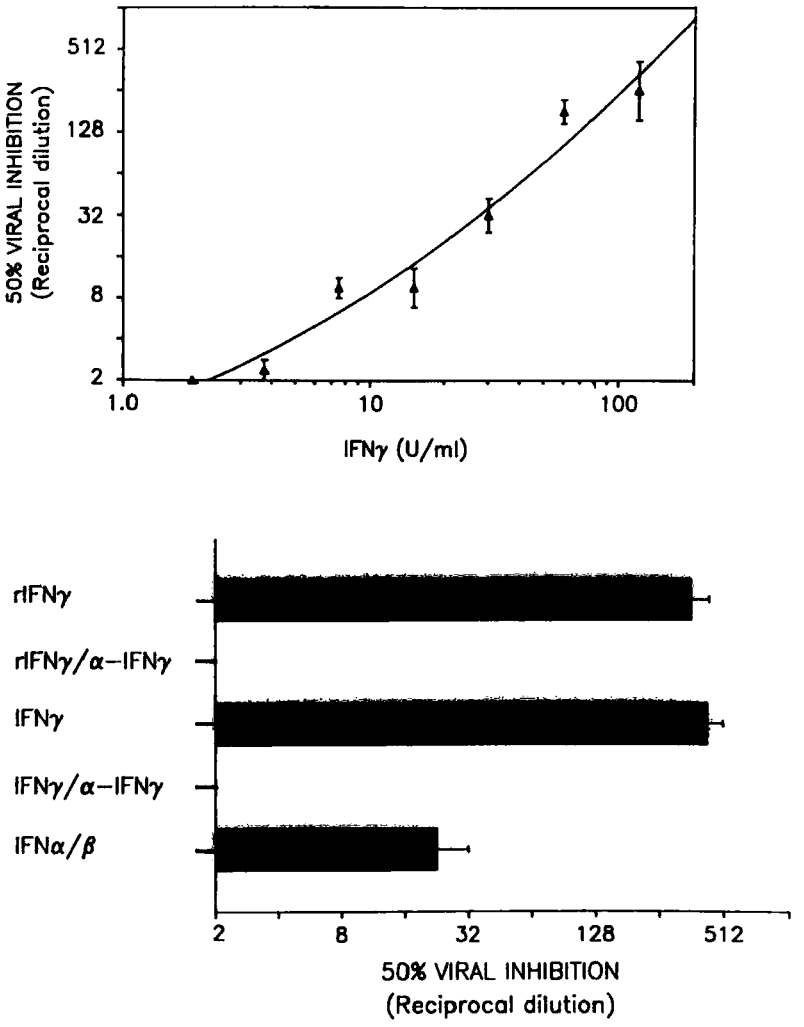

Figure 1. IFNγ quantitation by inhibition of viral cytopathic effect: Sensitivity (*top panel*). Specificity (*bottom panel*); viral inhibition mediated by natural and rIFNγ is neutralized by culture in the presence of anti-IFNγ mAb. The protection conferred by the addition of IFN α/β (100 U/ml) was not affected by the addition of anti-IFNγ mAb (not shown). Data are presented as reciprocal titres ± SEM derived from one of five independent assays.

This assay offers the least precise approach of the three examined, yielding a titre based on 50% inhibition of viral cytopathic effect. As such, it provides a rather subjective analysis of IFNγ production. Its characteristics would make it inappropriate for rigorous applications such as limiting dilution analysis.

Inhibition of WEHI 279 Cell Growth

B cell proliferation is markedly sensitive to IFNγ production. This sensitivity has been exploited in assays based on the dose dependent inhibition of B hybridoma WEHI-279 proliferation (6). Using ^{3}H-thymidine uptake as described, this approach reproducibly quantifies IFNγ concentrations at and above 2 U/ml (Figure 2). Using the method described here, we found this assay to exhibit excellent sensitivity and assay to assay reproducibility. The measurement of natural or rIFNγ was not interfered with by addition of the functionally related (IFNα/β) or unrelated cytokines tested. However, addition of acidic, exhausted tissue culture supernatants elicited variable inhibition of WEHI-279 B cell growth which was not neutralizable by anti-IFNγ mAb. This non-IFNγ dependent inhibition limits the utility of this assay. A further disadvantage is that the sensitivity of the assay is exquisitely dependent on the WEHI-279 cells being in log phase growth, with 2-5 fold decreases in sensitivity resulting from use of cultures containing 3-5% non-viable cells. Finally, though this assay is not sensitive to the presence of T cell mitogens such as anti-CD3 or Con A, the presence of mitogens such as LPS or PHA in the samples renders the

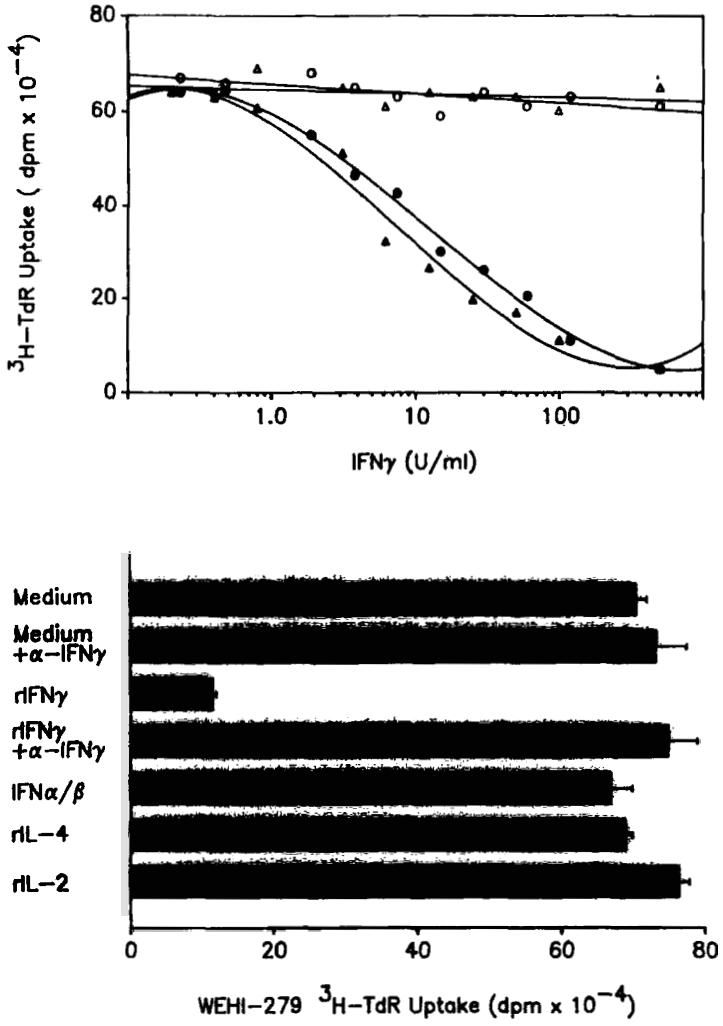

Figure 2. WEHI-279 growth inhibition by natural IFNγ (●○) and rIFNγ (▲△) in the absence (closed symbols) or presence (open symbols) of anti-IFNγ mAb: Sensitivity (*top panel*). In this assay, SEM ranged from 6-13% and are omitted for clarity. Specificity (*bottom panel*); Addition of rIFNγ (100 U/ml) but not IFN α/β (100 U/ml), rIL-2 (500 U/ml) or rIL-4 (500 U/ml), leads to inhibition of WEHI-279 proliferation which is reversible by the addition of anti-IFNγ mAb.

assay useless. In our hands, the variability encountered in ^{3}H-TdR uptake (SEM typically 5-8%), limit the utility of this approach for labor intensive, rigorous applications such as limiting dilution analysis, but do not detract from its usefulness in analysis of bulk culture supernatants obtained in antigen-driven T cell responses (8,11).

IFNγ ELISA Development

The inherent advantages of ELISAs prompted us to establish an IFNγ specific, dual mAb based assay using two mAbs which recognize spatially distinct epitopes on natural IFNγ. The assay can be performed in a single microtiter plate in 8-10 hours, although for convenience it is typically carried out over 24 hours. Unlike the bioassays, it does not require continuous culture of IFNγ responsive cells nor optimization of their growth prior to bioassay. It yielded the most consistent results of the three assays examined.

Using purified anti-IFNγ mAb XMG 1.2 and biotinylated R4-6A2 as capture and developing mAb respectively, this assay quantitates IFNγ from 0.5 U/ml to 100 U/ml. The specific upper and lower limits of the linear portion of the standard curve, hence the useful portion of the assay, are determined by the length of time the alkaline phosphatase/substrate step is allowed to proceed. The slopes of the linear portions of the curves generated with natural IFNγ (Figure 3A), rIFNγ (not shown) and a large number (>200 18 hour primary tissue culture supernatant samples) (8,11) was almost invariably parallel, an essential requirement for analyte quantitation.

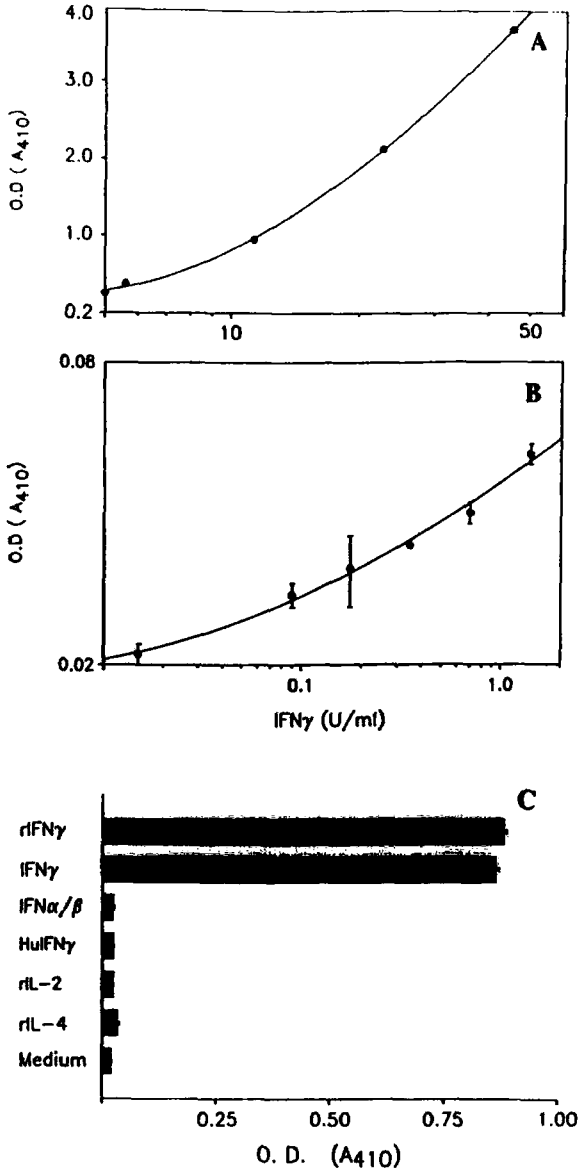

Figure 3. IFNγ determined by dual mAb ELISA: *Panel A, B.* ELISA sensitivity at IFNγ concentrations from 0.1 U/ml to 50 U/ml. Data shown are ± SEM. Note expanded Y axis in Panel B. *Panel C.* Specificity: Addition of IFNα/β (100 U/ml), HuIFNγ (500 U/ml), murine rIL-2 (500 U/ml) or rIL-4 (500 U/ml) failed to result in a detectable signal. Natural and rIFNγ were used at 10 U/ml in this assay. Data shown are ± SEM.

As described here, this assay yields extremely small standard errors, frequently <1%. Thus, the *detection* limit of the assay, defined as three standard deviations above the A_{410} of control wells lacking only IFNγ, is commonly 0.05 to 0.1 U/ml (Figure 3B), equivalent to 0.1 pg/ml or 5 x 10^{-15} M based on a Mr of 20 kDa for IFNγ (15). This highlights the advantages of this assay in applications such as limiting dilution analysis in which hundreds of small volume samples, many containing low levels of cytokine, must be analysed.

This assay also has the advantage of being much more rapid and independent of interference from other cytokines or non-specific variables (i.e., waste metabolites in culture supernatants) than the two bioassays tested. The sole limitation of this assay, in common with all ELISAs, is that it does not directly assess biological activity of the analyte.

Statistical Analyses

Intra-assay variation was determined following quintuplicate analysis of rIFNγ and natural IFNγ standards in each of the three assays. As can be seen from Table 1, a major advantage with the ELISA was that it exhibited the lowest variability of the three assays examined. The mean intra-assay coefficient of variation for the ELISA was 0.077, approximately one fifth of those obtained in the two bioassays. Similarly, comparison of the interassay coefficient of variation for the three assays tested identifies the ELISA as the assay of choice (Table 2).

TABLE 1

Intra-assay Variation in IFNγ Determinations

IFNγ added (U/ml)	ELISA Mean (C.V.)	WEHI assay Mean (C.V.)	Viral inhibition Mean (C.V.)
60	59.8 (.031)	77.7 (.299)	48 (.33)
30	32.0 (.073)	33.9 (.352)	21 (.390)
15	13.6 (.125)	13.3 (.462)	18 (.370)
7.5	6.8 (.090)	8.5 (.462)	6 (.340)
3.75	3.1 (.068)	2.2 (.358)	4.5 (.660)
1.88	1.88 (.069)	2.7 (.825)	N.D.

Legend: Cytokine concentrations were calculated from data obtained in the ELISA, WEHI-279 growth inhibition and inhibition of viral cytopathic effect assays carried out as described at Materials and Methods. For each IFNγ concentration, the mean of five replicates (and the associated coefficient of variation) is provided. N.D., Not done as this concentration was below the level of detection in this assay.

TABLE 2

Inter-assay Variation in IFNγ Determinations

IFNγ added	ELISA	WEHI assay	Viral inhibition
(U/ml)	Mean (C.V.)	Mean (C.V.)	Mean (C.V.)
60	57.5 (.121)	51.8 (.430)	45.8 (.260)
30	33.5 (.076)	24.4 (.364)	23.3 (.194)
15	12.1 (.145)	13.1 (.217)	18.4 (.168)
7.5	5.4 (.124)	6.1 (.331)	7.3 (.265)
3.75	3.0 (.119)	3.1 (.369)	1.4 (.289)
1.88	1.7 (.678)	1.8 (.851)	N.D.

Legend: The mean cytokine concentration and coefficient of variation obtained from four replicates of each assay at each IFNγ concentration is shown.
N.D., not done.

DISCUSSION

The spectrum of biological activities exhibited by IFNγ allows a wide choice of parameters which can be measured for IFNγ determinations. Most common bioassays evaluate viral inhibition, inhibition of cell growth or increased MHC class II antigen expression. However, all bioassays have rigid requirements in that to obtain useful sensitivity, the cells must be virtually 100% viable and in log phase growth. We have compared the advantages of the three most common assays currently in use for murine IFNγ determinations.

Bioassays measuring inhibition of viral cytopathic effect or inhibition of B cell proliferation offer good sensitivity and limited inter-assay variability but are more labor intensive, susceptible to day to day differences in the target cells of the bioassay, and require a moderate level of proficiency to maximize their reliability. In contrast, this IFNγ ELISA is readily established using widely available reagents or cell lines and offers greatly decreased assay times as well as increased simplicity and sensitivity.

Since the curves obtained with primary tissue culture supernatants were usually parallel in slope to those obtained with naturally produced or recombinant, CHO derived IFNγ, these internal standards are readily used to quantify the natural product in the WEHI-279 bioassay or ELISA. The nature of the end point in the inhibition of viral cytopathic effect bioassay (50% inhibition of viral cytopathic effect) does not support reliable standard curve analysis. The ELISA and WEHI-279 assays are sufficiently sensitive

to readily quantitate IFNγ production in antigen driven cultures (8,11).

Use of an MTT tetrazolium colorimetric assay in place of tritiated thymidine incorporation can increase the sensitivity of the WEHI-279 bioassay by up to 5-10 fold (12; our unpublished data). This variant benefits significantly from decreased assay time, an absence of radioisotopes and decreased materials and labor costs. However, it does not address the persistent concern of all bioassays with respect to potential misinterpretations arising from the interaction of multiple cytokines on the bioassay cell. Moreover, in the analysis of large numbers of exhausted tissue culture supernatants we have found that non-specific factors, not neutralizable by anti-IFNγ mAb, can act to inhibit bioassay cell proliferation, leading to false positives.

An alternative ELISA for murine IFNγ, based on capture with anti-IFNγ mAb R4-6A2 and use of biotinylated polyclonal rabbit antiserum, streptavidin-enzyme conjugates as developing reagents, has been described (14). The limitation of this approach lies in the requirement for a source of a polyclonal antibody highly specific for IFNγ or micro- to milligram quantities of purified IFNγ for immunization. These considerations have acted to limit its use.

In summary, after use of these bioassays for several years, we believe that development of this readily established sandwich immunoassay make it the optimal tool for determining murine IFNγ responses at physiological levels.

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