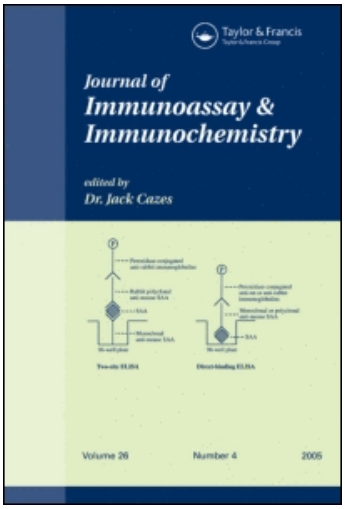

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Chemiluminescent and Enzyme-Linked Immuno Assays for Sensitive Detection of Human IFN-γ

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CHEMILUMINESCENT AND ENZYME-LINKED IMMUNO ASSAYS FOR SENSITIVE DETECTION OF HUMAN IFN-γ

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ABSTRACT

We have produced and characterized 4 mAbs to human IFN–γ and established sensitive, non-radioactive immuno-assays. The first two assays use microtiter plates as the solid phase and enzymes or chemiluminescence (acridinium ester) for development. The use of chemiluminescence instead of peroxidase increased the sensitivity of the assay by a factor of about 75. The third and the fourth assays utilize polystyrene beads as the solid phase and enzymes or acridinium ester for development. The use of beads also increased the sensitivity of detection. The most sensitive IFN–γ detection was achieved by the combination of bead with acridinium ester. In this configuration we were able to detect about 0.2 pg/ml IFN–γ (1/250th of a unit). These chemiluminescent immunoassays (CLIA) appear to be more sensitive than existing ELISAs or radioimmunoassays and may find new application areas.

(KEY WORDS: Interferon-γ, Chemiluminescence, ELISA)

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INTRODUCTION

IFN–γ is a glycoprotein produced by natural killer (NK) cells, CD4 positive T helper type 1 cells and CD8 positive cytotoxic T cells upon antigenic or mitogenic stimulation. Owing to its ability to activate macrophages and to induce MHC class II antigen expression, IFN–γ plays an important role in protection against infection by intracellular pathogens such as viruses and bacteria (reviewed in 1-3). Furthermore, numerous studies indicate a role for IFN–γ in several inflammatory and autoimmune diseases (1, 4-7), delayed type hypersensitivity (8) and AIDS (9-12). Recently, IFN–γ has been found to antagonize the IL-4 mediated immunoglobulin switch of B cells to expression of IgE, and IFN–γ may therefore be crucial in limiting allergic immune responses (13-15, reviewed in 16-18).

These rapid developments intensified the interest in the role of human IFN–γ (huIFN–γ) in immunoregulation and in the therapeutic potential of IFN–γ, IFN–γ agonists or antagonists. Although several IFN–γ immunoassays have been developed (19-31) there is still a need for improved IFN–γ assays particularly when early kinetics of IFN-γ production need to be studied.

We recently produced several mAbs against IFN–γ. Most of these potently neutralized the biological activity of IFN–γ. We show here that among four mAb based assay variations, immunoassays based on chemiluminescence were the best in terms of sensitivity, reproducibility and working range.

MATERIALS AND METHODS

IFN Preparations

Three different recombinant human IFN–γ preparations were used; IFN–γ from Kyowa Hakko (Japan) contained the N-terminal tyr-cys-tyr sequence and had two amino acid differences at positions 10 and 140 (32). This IFN–γ preparation had a specific antiviral activity of 1.8 x 10^7 IU/mg protein. A second IFN–γ was a product of Biogen (Geneva) kindly provided by Dr. M. Aguet (Zurich). A third IFN–γ preparation was kindly provided by Hoffmann-La Roche, Basle. Both of these preparations had a specific activity of 2 x 10^7 IU/mg protein. IFN-α and IL-4 used as controls were produced at the Biotechnology Department of Ciba-Geigy (Basle) as previously described (33). Recombinant mouse IFN–γ was a kind gift from Dr. G.R. Adolf (Ernst-Boehringer, Vienna) and had a specific activity of 1 x 10^7 IU/mg.

Production and Characterization of mAb to hu IFN–γ

Two BALB/c mice (10-14 weeks old; Sisseln, Ciba-Geigy, Switzerland) were injected with 10 μg recombinant IFN–γ (Kyowa Hakko) in PBS, intraperitoneally, every second week. After 4 such injections the mice were rested for 3 months and boosted i.p. with 25 μg IFN–γ in PBS.

Three days thereafter, spleen cells were fused to PAI myeloma cells (34) according to standard techniques (35, 36). Briefly, spleen cells (10^8)

of each mouse were fused with 10^7 PAI myeloma cells in 1 ml serum free medium containing 50% PEG 4000 (Merck) and distributed into 96 wells (4 x 24 well, Costar). From each fusion 96 growing cultures were obtained. After 3-4 weeks hybridoma supernatants were screened for anti-huIFN–γ activity using an ELISA based on two mAbs against IFN-γ and testing for inhibition of IFN-γ detection by hybridoma supernatants. Briefly, the capturing mAb 42-103 was fixed to microtiter plates. After blocking and washing, a simultaneous incubation with a mixture of IFN–γ, the second biotinylated mAb KM-48 and the test supernatant was carried out. After 3 h incubation and washing bound mAb KM-48 was revealed by streptavidin - alkaline phosphatase as described below. The mAb 42-103 was produced in collaboration with Dr. G. Delespesse (Montreal) and mAb KM-48 was a kind gift from Kyowa Hakko (Tokyo); while the latter mAb neutralized biological activity of IFN–γ the mAb 42-103 did not. The neutralization activity was determined as follows: 10 units of Biogen IFN–γ were incubated with 2 fold serial dilutions of hybridoma supernatants for 1 h at room temperature and antiviral activity was determined as described (37). Induction of ascites fluids, purification of mAbs and determination of the class of the Igs were performed according to standard methods (38). All the mAbs were of the IgG1K isotype.

Antiviral and Neutralization Assays

A cytopathic inhibition assay using WISH or Hep/2 cells with vesicular stomatitis virus as the challenge virus was used for both the determination of biological activity of IFN–γ (units/ml) and of the neutralization capacity

of the mAbs (33). For calibration we used the WHO IFN–γ standard (Cg 23-901-530 with 4000 IU/ml).

Determination of Affinity

The method using underivatized mAb or IFN–γ was adapted from Friguet et al. (39). In brief, fixed concentrations of IFN–γ (Kyowa Hakko 50 pg/ml) were incubated with varying concentrations of purified mAb for 68 h at 4° in assay buffer. These solutions were transferred to blocked microtiter plates coated with the same mAb, incubations were continued for 160 min. at room temperature to allow binding of uncomplexed (free) IFN–γ. Plate bound IFN–γ was finally revealed by suitable mAbs recognizing a second epitope as described above. Calculations were based on the law of mass action assuming a molecular weight of 17000 for IFN-γ and 80000 for one binding site of an antibody. Dissociation constants were derived by direct fitting of the raw data using the curve fitting facility of the Sigma-Plot software package (Jandel Scientific).

ELISAs for IFN–γ

A. Microtiter plate (conventional) ELISA (one-step procedure): For coating 100 µl of coating buffer (sodium bicarbonate buffer 0.1M pH 9.6, merthiolate 0.01%) or PBS (phosphate buffered saline pH 7.4) containing 5 µg/ml (e.g. 43-11) mAb was distributed into the wells of microtiter plates (Dynatech) and left overnight at 4° C. All subsequent steps were carried out at room temperature. The solution was removed by flicking and plates were washed 6 times with washing buffer (PBS containing 0.05% Tween

20). Microtiter wells were then blocked for 1 h with assay buffer (washing buffer plus 1% bovine serum albumin and 0.01% merthiolate, 200 μl/well). Fifty μl of mAb 45-11 coupled to biotin diluted to 1 μg mAb/ml in assay buffer were added to each well followed by 50 μl of IFN–γ standard or test samples. The plates were incubated at room temperature for 2 h. The plates were flicked, washed 6 times with washing buffer and incubated with an extravidin-peroxidase conjugate (Sigma, 2 μg/ml in assay buffer) for 1 h. After washing 6 times, 150 μl of substrate solution (1.5 mg/ml o-phenylene diamine (OPD) in citrate buffer pH 5.5, supplemented with 0.03% H_2O_2 before use) was distributed to each well. The reaction was stopped after 10 min by adding 50 μl of 0.5 N H_2SO_4. The optical density at 490/630 nm was determined by a computerized ELISA reader (Biorad Titertech Multiscan). The amount of IFN–γ present in the samples was calculated by reference to a standard curve. All samples were run in triplicate.

B. Bead-ELISA (two-step procedure): Polystyrene beads (6.35 mm diameter, Spherotech, Zürich) were stirred with 70% ethanol and washed with PBS. MAb (43-11) was used for coating (5 μg/ml in PBS) overnight at 4 °C. The beads were then blocked in assay buffer for 1 h at room temperature and washed 3 times. They were incubated for 2 hours with 400 μl samples and standards in 48 well plates or in 5 ml tubes. After washing, 400 μl biotin-labelled mAb 45-15 (1 μg/ml in assay buffer) was added to the wells or tubes and incubation continued for 1 hour. After washing and a further 1 h incubation with 400 μl of extravidin-peroxidase (Sigma, 2 μg/ml in assay buffer) the colour reaction was started. The substrate solution (400 μl) was 1.5 mg/ml OPD, 0.03% H_2O_2 in citrate

buffer pH5.5. The reaction was stopped by adding 100 µl 0.5 N H_2SO_4 and the optical density determined on a ELISA reader (Titertech Multiscan) after transferring 200 µl aliquots to 96 well plates.

Chemiluminescence Immuno Assays (CLIA)

The mAb 45-15 was labelled with DMAE-NHS (2,6,-dimethyl-4-(N-succinimidyloxycarbonyl)phenyl 10-ethylacridinium-carboxylate methosulfate, a kind gift of Dr. S.-J. Law Ciba-Corning Diagnostics) (40) according to Weeks and Woodhead (31). In brief, 50 µg mAb in 150 µl 0.1M NaH_2PO_4, 0.15 M NaCl (adjusted to pH 8 with NaOH) were incubated at room temperature with 20µg DMAE-NHS (dissolved in 20 µl DMSO). After 20 min the reaction was quenched with 200 µg lysine and the antibodies were isolated by fractionation on 5 ml of Biogel P-6 (Biorad).

Chemiluminescence was determined by injection of 0.5% peroxide in 0.1N nitric acid and 0.25N NaOH containing detergent. Depending on the assay, an instrument measuring chemiluminescence in single tubes (Luminomat II, Ciba-Corning Diagnostics) or in microtiter plates (LB96, Berthold, Wildbad,Germany) was used.

A. Microtiter-CLIA (one-step procedure): White plates (Microlite, Dynatech) were coated as described above for the microtiter plate ELISA. Further processing was analogous to this ELISA except that DMAE labelled antibody (final concentration of mAb 45-15, 0.3 µg/ml) was added to the samples (final volume: 100 µl). The plates were washed twice with washing buffer and once with water before determination of chemiluminescence in the microtiter plate luminometer.

TABLE 1

Properties of Monoclonal Antibodies and Suitability for Two-site Assays

			Second mAb			
Coating mAb	**Neutralization [a)]**	**Kd [pM]**	**43-11**	**23-9**	**45-15**	**76-18**
43-11	No	600	+	+++	++++	++
23-9	Yes	7	+++	+	++	++++
45-15	Yes	25	++	-	-	-
76-18	Yes	50	++	+++	++++	++
42-103 [b)]	No	2000	+++	-	+	+++

The suitability was assessed with biotinylated second antibodies using the plate-ELISA as described in the text. [a)] determined by inhibition of antiviral activity of IFN-γ. [b)] The mAb 42-103 was produced in collaboration with Dr. G. Delespesse in a separate experiment.

B. Bead-CLIA (2-step procedure): This was performed as described for the bead-ELISA except that 200 µl of second mAb 45-15 labelled with DMAE (0.3 µg/ml) were put into the tubes for 1 h. The beads were washed as above and chemiluminescence was determined on a single tube instrument as detailed above. MAb coated beads could be stored in the assay buffer at 4°C for at least 2 months.

T cell stimulation for IFN–γ production

Peripheral blood mononuclear cells (PBMC) were prepared from heparinized venous blood (10 U/ml, Liquemine, Roche) by the standard Ficoll-Hypaque (Pharmacia) method.

The cells were washed 3 times with PBS and resuspended in RPMI 1640 culture medium supplemented with 10% fetal calf serum and

penicillin (100 units/ml) and streptomycin (100 μg/ml, all from Gibco). For the whole blood assays, venous blood was diluted 1:4 with the above medium. Viability of cells was > 80% as determined by the fluorescein diacetate method. The following T cell stimulating agents were used; phytohemaglutinin (PHA, 1μg/ml), anti-CD3 mAb (OKT3, Ortho Diagnostics, coated at 1 μg/0.1 ml/well) and purified protein derivative of M. tuberculosis (PPD, 1 μg/ml, Serum Institute, Kopenhagen). Cells (10^5/0.2 ml/well microtiter plate, Dynatech) were incubated at 37°C, 7% CO_2, in air; supernatants were collected at indicated time points and frozen at -80°C until testing.

RESULTS

Properties of mAbs

From two fusion experiments 192 growing cultures were obtained; in 16 cultures mAb against human IFN–γ were detected. Four hybridomas from four different cultures (23-9, 76-18, 43-11, 45-15) were isolated by limiting dilution cloning, expanded and their mAbs characterized. Competition binding to radiolabelled IFN-γ indicated that mAbs 43-11 and 76-18 recognized similar epitopes whereas mAbs 45-15 and 23-9 recognized a second epitope.

In biological tests all except one (43-11) inhibited the antiviral activity of IFN-γ (Table 1). Interestingly, all neutralizing mAbs exhibited higher affinity (Kd: 7-50 pM) than non-neutralizing mAbs (Kd 500-2000 pM). The antibody dilution curves of the mAbs, 43-11 and 45-15 which were used throughout this study are shown in Fig. 1.

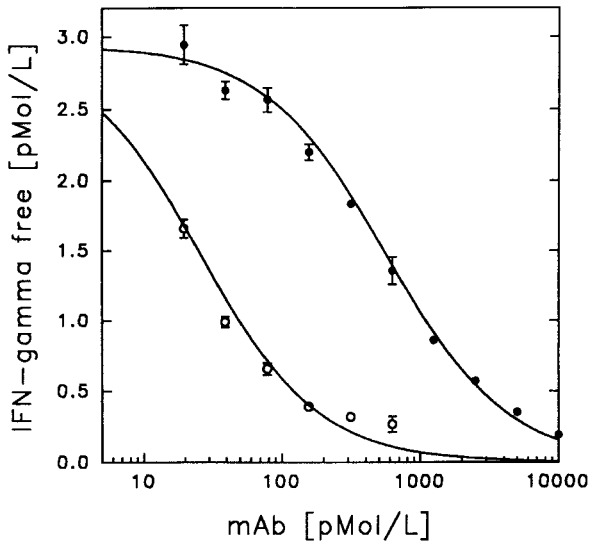

FIGURE 1. Affinity of monoclonal antibodies against IFN-γ. The symbols indicate the concentrations of free IFN-γ determined after equilibration in the presence of varying concentrations mAb 43-11 (filled circles) or mAb 45-15 (open circles). Curve fitting according to the law of mass action resulted in Kds of 600 pM (mAb 43-11) and 25 pM (mAb 45-15). Error bars indicate standard deviations of triplicates. For details see methods section.

Microtiter Plate-ELISA

In order to assess suitability for two-site assays each mAb was labelled with biotin and tested in combination with itself and with all other mAbs in a two-site solid phase ELISA. As shown in Table 1, several pairs of mAbs were found to detect hu IFN–γ. The observation that even a single mAb could be used for constructing sandwich-type immunoassays can be explained by the ability of IFN-γ to form dimers. However, in practice, these cannot be used because the signals obtained were too low. Two

TABLE 2

Immunoreactivity of IFN-γ after Denaturing and Cross-Reactions with other Cytokines

	(ng/ml)	Immunoreactivity [c]
Hu IFN-γ		100 %
Hu IFN-γ heat treated [a]	10	0.05 %
	100	0.05 %
Hu IFN-γ acid inactivated [b]	10	< 0.01 %
	100	< 0.01 %
Mouse IFN-γ	10	< 0.01 %
	100	<0.01 %
Human IL-4	10	< 0.01 %
	100	< 0.01 %
Human IFN-αB	10	< 0.01 %
	100	< 0.01 %

[a] 56°C, 2 h,[b] pH 2.2, 18 h,[c] as determined on a standard curve in assay buffer.

pairs of mAbs were selected for further use (43-11/45-15 and 23-9/76-18). Superimposable standard curves were obtained with these pairs of mAbs. Their detection range was 0.05 to 10 ng/ml of hu IFN-γ. In the following we only present data obtained with the 43-11/45-15 pair. For optimizing the assay we varied the sample incubation time (1,2,3 or 4 h) and found negligible differences in sensitivity or in the shape of the curve. However, the sensitivity decreased when the incubation time was only 30 minutes.

As shown in Table 2 neither human IFN-alpha B, human IL-4 nor mouse IFN-γ produced any signal. Neither heat nor acid inactivated IFN-γ was detected by ELISA.

In order to assess quenching effects by human serum we added graded amounts of IFN–γ (Biogen) into 1:2 diluted normal human serum

and compared it with IFN–γ in buffer. In the linear range of the assay quenching was approximately 25 to 50%. In addition, we demonstrated that the ELISA could be used for the detection of in vitro produced IFN–γ in the plasma fraction of 1:4 diluted human whole blood (data not shown).

Comparison of Plate and Bead ELISA

The binding capacity of polystyrene beads with their large surface area was expected to exceed that of microtiter plates. Indeed, the signals obtained from the bead assay were considerably higher than in the microtiter plate version. This was particularly evident at low concentrations of IFN-γ; the resulting gain in sensitivity was about 3-fold. Fig. 2 shows standard curves obtained by plate v.s. bead-ELISA. Note that incubations in the bead assay were sequential whereas the microtiter assay was simultaneous.

Chemiluminescent Immunoassays (CLIA)

Acridinium esters can be used as labels of high specific activity which decay under emission of a flash of light when exposed to alkaline peroxide. mAbs are easily derivatized with the acridinium moiety and when analyzed with suitable instruments allow construction of sensitive assays. In the following set of experiments we investigated whether our IFN–γ sandwich assay would benefit from reformatting to this technology.

We first adapted the ELISA system by replacing the biotinylated mAb by a DMAE-labelled mAb. As can be seen in Fig. 3 in the plate-CLIA system the standard curve indicated a linear assay range of 0.004 ng/ml up to 8

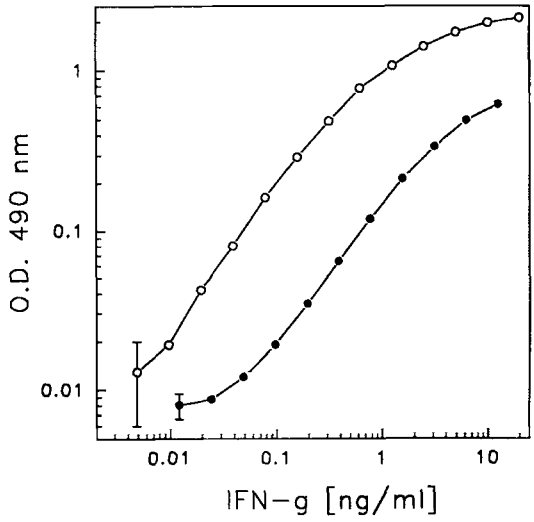

FIGURE 2. Standard curves for IFN-γ determination by ELISA using microtiter plates (closed circles) or polystyrene beads (open circles) as solid phases. In both assays, mAb 43-11 was immobilized on the solid phase and biotinylated mAb 45-15 served as second antibody. In the microtiter version first and second antibodies were simultaneously present; in the bead version, incubations were sequential. An avidin-peroxidase conjugate with o-phenylene diamine as substrate was used for the colour reaction. All determinations were done in triplicate; error bars indicate standard deviations. O.D. values of the blanks have been subtracted.

ng/ml with a limit of detection (4 times standard deviation) of 0.5 pg/ml. Interassay coefficient of variation was 13% at 0.5 ng/ml IFN-γ. Similarly, the bead (two step) ELISA system converted to acridinium ester labels (Bead-CLIA) improved the limit of detection to 0.2 pg/ml and extended the assay range from 0.001 ng/ml to up to 10 ng/ml (Fig. 3). Interassay coefficient of variation was 7% at 0.7 ng/ml IFN-γ. Although the luminometers used for these assays were both photon counting instruments the relative light units registered were lower in the microtiter reader. This was

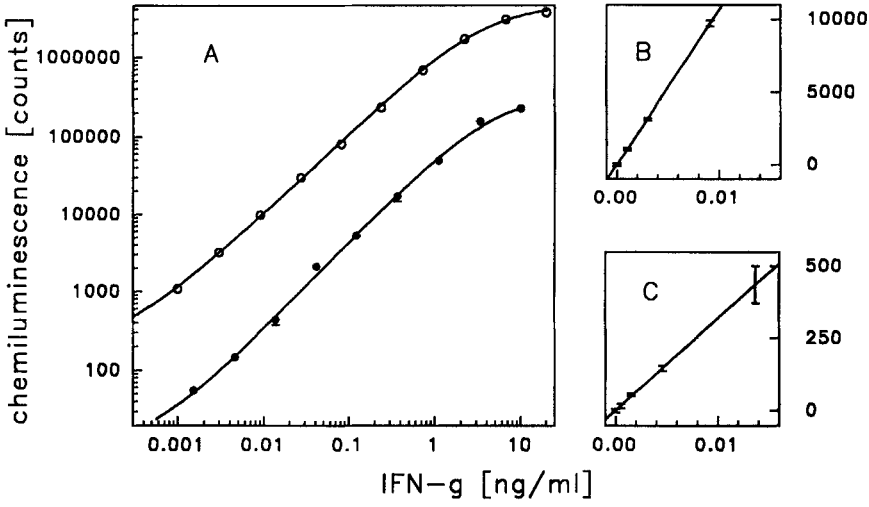

FIGURE 3. Panel A: Standard curves for IFN-γ determination by chemiluminescence immunoassay (CLIA) using microtiter plates with simultaneous incubation (closed circles) or polystyrene beads with sequential incubation (open circles). In analogy to the ELISA versions (cf. Fig. 2), mAb 43-11 was immobilized on the solid phase and DMAE-labelled mAb 45-15 served as second antibody. All determinations were done in triplicate; error bars indicate standard deviations. Backgrounds (60 and 830 counts for plate and bead assays, respectively) have been subtracted. Details of the curves at low IFN-γ concentrations are shown with linear scales in panel B for the bead assay and in panel C for the microtiter plate assay.

TABLE 3

Comparison of Chemiluminescent and Enzyme-Linked Immunoassays for IFN-γ

Assay type	Detection range (ng/ml)	Detection limit (pg/ml) [a]
1. Plate-ELISA	0.05 - 10	37
2. Bead-ELISA	0.02 - 10	15
3. Plate-CLIA	0.005 - 8	0.5
4. Bead-CLIA	0.001 - 10	0.2

[a] All at 4 x standard deviation above background. The figures were derived from 3 to 7 separate experiments.

TABLE 4

Kinetics of IFN-γ Production by T cells Detected by Bead-CLIA

IFN-γ [pg/ml]

	Experiment Number				
Hours	# 1	# 2	# 3	# 4	# 5
8	425 ± 15	15 ± 1	84 ± 3	27 ± 3	192 ± 17
24	962 ± 12	46 ± 0.5	220 ± 5	225 ± 45	512 ± 112
48	2950 ± 50	925 ± 125	1750 ± 150	6500 ± 750	3550 ± 550
72	NT	NT	9500 ± 500	>10.000	NT

Hu PBMC from different donors were stimulated with OKT3 mAb in microtiter plates and supernatants were collected at various time points. Exp. 3 and 5 used PBMC from the same donor at different times. Donor in experiment #2 produced low amounts of IFN-γ in two additional experiments. Background was less than 0.2 pg/ml. NT: not tested.

due both to differences of geometry and of detectors. Table 3 summarizes the detection ranges and sensitivities of the new assays.

Detection of IFN-γ Produced by Stimulated T Cells

As the mAbs had been raised against recombinant IFN-γ it was important to test natural IFN-γ in our assays. Natural IFN-γ is also known to differ from recombinant IFN-γ with respect to glycosylation and dimerization. We stimulated human T cells with an OKT3 mAb and determined IFN-γ levels by bead-CLIA at different time points. The results shown in Table 4 indicate that readily detectable levels of immunoreactive IFN-γ were produced. Striking donor-specific differences of IFN-γ production

were observed. For instance, one donor consistently produced less IFN-γ than the others. The plate-ELISA gave similar results.

DISCUSSION

In this study we describe non-radioactive immunoassays for IFN-γ which combine high sensitivity with short duration and large working range. We developed new high affinity mAbs and evaluated a selected pair in four assay types. Two solid phases (plates or beads) were combined with two detection systems (peroxidase or acridinium esters). The conventional plate-ELISA developed with the mAb pair 43-11/45-15 was comparable to other immunometric assays with regard to sensitivity and working range (20-23, 26, 27). This ELISA has now been used in hundreds of measurements over a period of one year and sensitivity has reproducibly been less than one unit/ml (< 50 pg/ml).

Changing the solid phase from plates to beads improved the ratio of signal to noise 2- to 3-fold. The use of acridinium ester instead of enzymes drastically increased sensitivity of detection by a factor of 75. Thus, we showed that the bead-CLIA could reproducibly detect 0.2 pg/ml IFN-γ. Since the sensitivity of most bioassays and immunoassays is usually around one unit/ml, this represents a 250-fold gain in sensitivity. To our knowledge this constitutes the most sensitive immunometric assay for IFN-γ. IFN-γ is readily detectable by available assays in long term cell cultures. However, the high sensitivity of our new CLIA assays is essential for studying early kinetics of IFN-γ production upon T cell activation in culture. Here, the bead-CLIA is particularly useful. We have recently shown that human PBMC cultures produced considerable amounts of IFN-γ as

early as 2 to 4 h after stimulation (Akdis et al., in preparation). Furthermore, preliminary results indicate that IFN-γ production by T cells stimulated by physiological agents such as antigens (e.g. PPD and tetanus toxoid) can be studied in human PBMC in vitro.

Beads have previously proven to be useful solid phases for commercially available radioimmunoassays and ELISAs. Although our new combination of beads with acridinium ester-labelled mAb brought several advantages such as low background, high signals and good reproducibility, they have some drawbacks: standard beads are of 6.2 mm diameter and thus the assays can only be performed in 48 well plates or tubes. This is an obvious disadvantage for studies with many samples. Here, our plate-CLIA assay which is over 50-fold more sensitive than conventional plate-ELISA is ideal (see Table 3). On the other hand, beads as solid phase for the first mAb offer additional possibilities of application. One of these is trapping of IFN-γ by a mAb-coated bead present during the culture for subsequent immunometric detection preferentially with acridinium ester-labelled mAbs. Using such systems we are currently studying the kinetics of IFN-γ consumption and/or degradation in culture. Furthermore, we are investigating similar assays for other cytokines such as IL-1 and IL-4.

The newly developed immunoassays may find application in systems where few cells, such as organ biopsies, produce small amounts of cytokines. In the light of recent exciting findings on the cross-regulation of TH_1/Th_2 cells (16-18) study of the kinetics of IFN-γ and IL-4 production may be crucial for understanding the pathogenesis of autoimmune diseases (1,4-7), allergy, asthma (13-18) and AIDS (9-12).

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