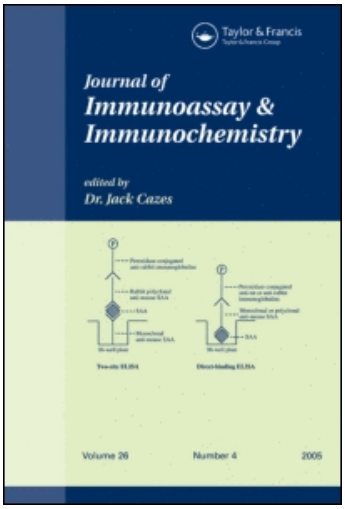

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INCREASED SENSITIVITY OF AN ENZYME IMMUNOASSAY FOR HUMAN INTERFERON ALPHA 1 USING A MIXTURE OF THREE MONOCLONAL ANTIBODIES

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ABSTRACT

A sensitive sandwich ELISA for the quantification of human interferon (IFN)-α1 was designed. The assay employed IFN-α1- specific polyclonal antibody for coating and monoclonal antibodies (mAbs) to IFN-α 1 as a second antibody. A major increase in sensitivity of the assay could be achieved, when polyclonal antibody was combined with a mixture of three mAbs binding to different regions of IFN-α1, compared to the combination of a polyclonal antibody with a single mAb. The sensitivity of the established ELISA was close to that of an antiviral IFN-bioassay. The ELISA did not cross-react with IFN-α2 , β,τ or omega. The immunoassay allowed to estimate the content of IFN-α1 in leukocyte IFN-α to about 25-50% or to 2-6% in Namalwa IFN-α , respectively.(KEY WORDS: ELISA, Interferon α1, Monoclonal antibody)

INTRODUCTION

Among 15 related subtypes of human interferon (IFN)-α, the subtypes IFN-α1 and IFN-α2 are considered

of the major importance, because they represent the main species of natural IFN-α (1,2). There were developed several enzyme-linked immunosorbent assays (ELISA) which are able to measure human IFN-α, however, they cross-react with IFN-α1 and IFN-α2 (3-6).In this paper we described the specific sandwich ELISA for the detection and quantification of recombinant or natural human IFN-α1. The ELISA was based on IFN-α1-specific sheep polyclonal and murine monoclonal antibodies. We showed, that a combination of the first (polyclonal) antibody with the mixture of different monoclonal antibodies (mAbs) to IFN-α1 resulted in a major increase in sensitivity of the assay, compared to the test-sytem using a combination of a polyclonal antibody with an individual mAb.

MATERIALS AND METHODS

Interferons

Recombinant human IFN-α1, IFN-α2c and IFN-omega 1 were kindly provided by Prof. G. Bodo (Boehringer, Vienna, Austria). Recombinant human IFN-β was kindly supplied by Bioferon (Laupheim, Switzerland) and recombinant human IFN-τ was a gift from Prof. V.P.Zav'yalov (Institute of Immunology, Lyubuchany, Russia).

Antibodies to Human IFN-α1

Polyclonal antibodies were produced by immunization of a sheep with recombinant IFN-α1. The immunoglobulin (Ig) fraction of sheep antisera was isolated on DEAE-cellulose (7).

Preparation and characterization of the mouse mAbs 1-9, 1-38 and 2-48 (IgG1-subclass) raised to recombinant IFN-α1, was described previously (8). These neutralizing antibodies recognized three different antigenic sites on IFN-α1 molecule. The epitope for mAb 1-9 was located into region 63-67, mAb 1-38 bound to the segment around residues 43-53, and the sequence 63-76 formed the epitope for mAb 2-48.

IFN-Bioassay

The antiviral activity of IFN was determined by the cytopathic effect inhibition method using human A549 cells challenged with vesicular stomatitis virus in microtitre plates (9). The assay was calibrated against standard of human leukocyte IFN-α G 032-901-527, supplied by the National Institute of Healths (Bethesda, MD).

IFN-α1-Sandwich ELISA

Immunoplates (Koh-i-noor, Czecho-Slovakia) were coated with 50 μl per well of sheep anti-IFN-α 1-Ig diluted 1:4000 in phosphate-buffered saline (PBS;

pH 7,2) and incubated overnight at 37°C. After blocking with 1% nonfat dry milk, the different concentrations of IFN diluted in PBS were added in 50 µl amounts for 1 h at 37°C. Plates were washed and incubated with 50 µl/well either of a single supernatant or a mixture (1:1:1, v/v) of three supernatants for 1 h at 37°C. The bound monoclonal antibodies were detected with peroxidase-conjugated rabbit anti-mouse Ig (DAKO). After washing the raction was developed with orthophenylene diamine and absorbance at 492 nm was measured using Multiscan MCC/340 (Labsystems, Finnland). Absorbance value of a negative control (nonspecific binding; wells containing dilution buffer instead of IFN) was substracted from readings.

RESULTS

Polyclonal and Monoclonal Antibodies to Human IFN-α1

The polyclonal immunoglobulin (Ig)- fraction of sheep antisera after immunization with recombinant IFN-α1 was isolated. Purified Ig neutralized 10 units/ml of IFN-α1 at the dilution 10^{-6} (not shown).

Mouse monoclonal antibodies, designated 1-9, 1-38 and 2-48, with the high specific binding ability towards recombinant IFN-α1 were directed to three

distinct epitopes (8). The mAbs as hybridoma culture supernatants were used for experiments. The Ig-concentration in supernatants ranged between 0,4-0,5 μg/ml (8).

Establishment of a Sandwich ELISA

Human recombinant IFN-α1 in ten-fold dilution was titrated on the plates coated with the polyclonal sheep anti-IFN-α1 Ig. Then individual mAbs 1-9, 1-38 and 2-48, respectively, were compared as a second antibody for measuring of captured IFN. However, tested antibodies were able to generate similar titration curves for IFN-α1 within the range 10-10 000 units/ml (Fig.1).

Improvement in Sensitivity of the Sandwich ELISA

We supposed that three antibodies recognizing distinct sites could bind simultaneously to IFN-α1 molecule and thus to intesify the signal in our ELISA-system. To prove this possibility, supernatants 1-9, 1-38 and 2-48 were mixed at a ratio 1:1:1 (v:v) and this mAb-cocktail was used as the second antibody. The resulting standard curve of IFN-α1 (Fig. 1) demonstrated a significant (about 10-fold) increasing in the sensitivity of the assay. As little as 1 units/ml of IFN-α1 could be detectable in less than 5 h.

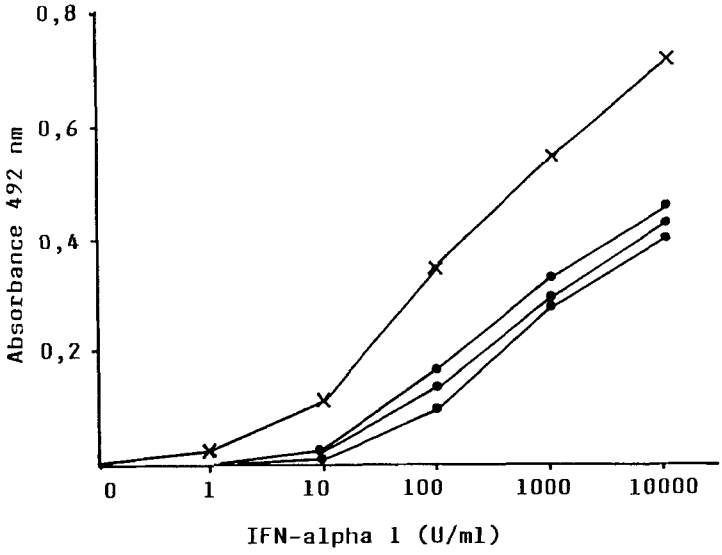

FIGURE 1. Standard curves for human IFN-α1 in sandwich ELISA using as a second antibody either single mAb (-o-) or cocktail of three mAbs (-x-). Means of quadruplicate determinations are shown. Standard deviation was less than 10% of the mean.

Specificity of the Sandwich ELISA

The assay was specific for recombinant human IFN-α1 and did not detect recombinant human IFN-α2, IFN-β, IFN-τ or IFN-omega 1 , each at the concentration 10^4 U/ml (Table 1). To examine the correlation between the sandwich ELISA and an antiviral IFN-bioassay, samples containing different amounts of IFN-α1 were titrated in parallel by both methods. A good correlation (0,9502) between the assays was found (Fig.2).

TABLE 1

Specificity of the Sandwich ELISA

Recombinant human IFN (10^4 units/ml)	Absorbance[a] at 492 nm
IFN-alpha 1	0,72
IFN-alpha 2	0,06
IFN-omega 1	0,07
IFN-beta	0,08
IFN-gamma	0,07

[a] Mean from four parallel wells.

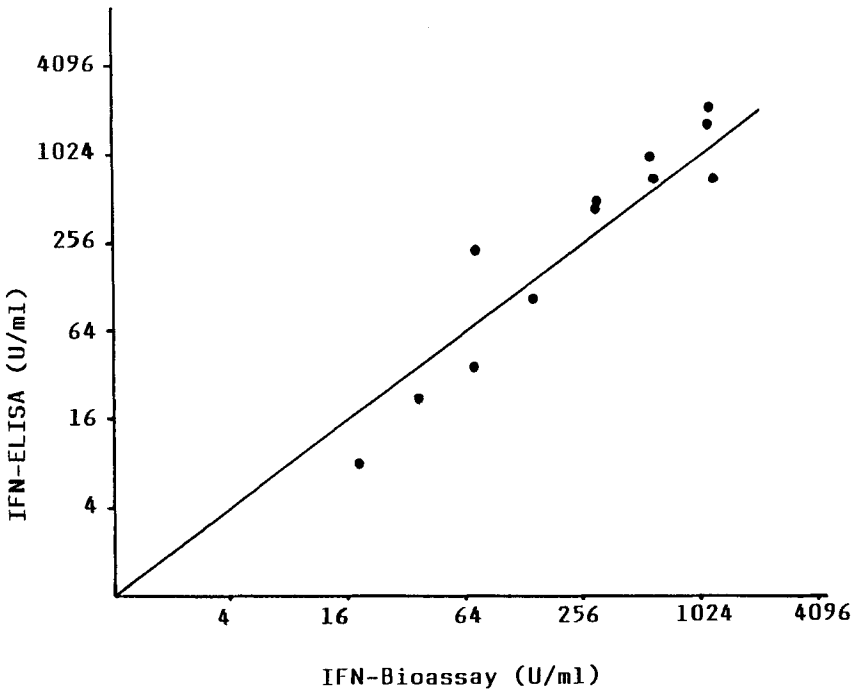

FIGURE 2. Correlation of IFN-α1 concentrations measured by antiviral bioassay and immunoassay (sandwich ELISA). Samples containing recombinant IFN-α1 were assayed by both methods. The results were correlated by linear regression analysis (r=0,9502).

TABLE 2

Relative Frequency of IFN-α1 in Natural IFN-α

Sample	IFN-α[a]	Antiviral[b] IFN-α titer (IU/ml)	ELISA[c] IFN-α1 titer (IU/ml)	Relative frequency of IFN-α1
1	leukocyte	512	256 (301±28)	50%
2	leukocyte	512	128 (200±17)	25%
3	Namalwa	1024	64 (85±7)	6%
4	Namalwa	512	8 (12±2)	2%

[a]Induced with Newcastle disease virus.
[b]Mean from triplicate determinations.
[c]Determined ELISA-titer in parenthesis (mean±SD from four parallel) was arranged according to the accuracy of antiviral bioassay (a multiple of log 2).

Measuring of the Natural Human IFN-α1

We used the established immunoassay for determination of the relative frequency of IFN-α1 in preparations obtained from human leukocytes and lymphoblastoid cells Namalwa stimulated with Newcastle disease virus. The antiviral titres of 2 samples of leukocyte IFN and 2 samples of lymphoblastoid IFN were compared with the corresponding ELISA-titres (Table 2). To allow an adequate calculation, ELISA-data (Table 2, in parenthesis) were arranged to fit in with the accuracy of bioassay, in which samples were titrated in two-fold dilution. For example, the Sample 1 with the ELISA-titer 301 should, theoretically, exert

an antiviral effect at the dilution 1:256 (>1 unit), but not at the next one 1:512 (<1 unit). Therefore in Table 2, ELISA-titer of this sample was considered as 128, etc. Using this attitude we assumed, that IFN-α1 could contribute with an about 25-50% to the total antiviral activity of human leukocyte IFN-α, whereas in lymphoblastoid IFN this subtype represented only 2-6%.

DISCUSSION

In spite that IFN-d1 is one of the most abundand subtypes present in natural IFN-α, a quantitative ELISA specific for this species has not been described. We developed a sandwich ELISA for specific measurement of IFN-α1 based on combination of polyclonal antibody to IFN-α1 with three mAbs to IFN-α1. In contrast to combination of polyclonal antibody with a single mAb, a significant increasing (about 10-fold) in the sensitivity of assay could be achieved, when polyclonal Ig for coating was combined with a cocktail of three mAbs to the distinct sites of IFN-α1. The enhanced sensitivity of ELISA could be explained by simultaneous binding of three different mAbs to the one captured IFN-α1 molecule (9,10). At this configuration, the assay was able to detect as little as 1 units/ml of IFN-α1. The ELISA, in contrast to bioassay, permits

a rapid and specific assessment of IFN-α1 also in preparations of natural IFN-α. Because of low frequency of other (not tested) α-subtypes in such preparations, theoretical cross-reactivity with some of them should not substantially affect the specificity of ELISA-determination. Therefore we estimated that IFN-α1 might be responsible for 25-50% of the total antiviral activity of leukocyte IFN-α, in contrast to the corresponding hardly 6% in lymphoblastoid IFN.

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