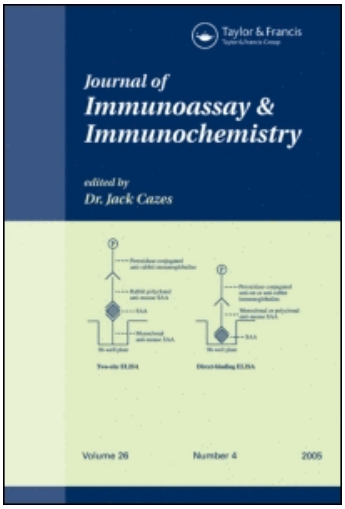

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Development and Validation of an Enzyme-Linked Immunosorbent Assay (ELISA) for Recombinant Human Gamma Interferon

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ABSTRACT

A one-site ELISA for the quantification of recombinant human gamma interferon (rh-IFN-γ) was developed and validated. A single monoclonal antibody (Mab) was used as a "catching" antibody and as a horseradish peroxidase (HRP)-labeled conjugate. Detection limit and quantification limit of this assay were estimated to be 1.26 and 15 ng/mL, respectively, and the coefficient of variation was below 15%. The ELISA was specific for rh-IFN-γ, showing no cross reactivity to other related molecules in the range of the concentrations studied. The results correlated well with those obtained by a bioassay method.

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By using this assay, it was demonstrated that 0.01–1% (v/v) Tween 80 protected rh-IFN-γ during freezing and thawing.

Key Words: ELISA; Gamma interferon; Monoclonal antibody.

INTRODUCTION

Gamma Interferon (IFN-γ) is a protein produced by activated T cells; it shows antiviral, antiproliferative, and immunoregulatory properties.[1] Through the expression of this protein in Escherichia coli and other genetically modified hosts,[2–4] significant quantities of this product have been obtained with a high purity. The rh-IFN-γ is effective in the treatment of juvenile chronic arthritis, chronic granulomatous disease, and in some infectuous diseases such as malaria and tuberculosis.[5–7]

For some years, various groups have reported the use of immunochemical systems, such as ELISA and immuno-radiometric assay (IRMA), for the quantification of very small quantities of this protein because they are very sensitive and less laborious than bioassays.[8,9] Some authors have used the capture and detection antibodies that recognize different epitopes of the molecule in immunoenzymatic assays,[10] while others have used the same antibody acting as capture and detection molecules for the quantification of human IFN-γ in serum.[11]

Human IFN-γ is a homodimer, a requirement for its biological activity.[12] Keeping in mind the characteristics of the IFN-γ molecule mentioned before, we have developed and validated an ELISA system to quantify the active protein, using only one monoclonal antibody as a capture and detection antibody. We also have investigated its correlation with bioassay and its application in formulation studies and in-process control.

EXPERIMENTAL

Materials

The rh-IFN-γ[2] used as standard was characterized by reverse phase HPLC at more than 99% purity and by gel filtration HPLC at more than 98% purity. The molecular weight obtained corresponded with that reported for the dimer, (31 kDa).[13] The monoclonal antibody CB-IFNG.100 against rh-IFN-γ (G 1 subclass Mab), the conjugates CB-IFNG.100-HRP and anti-mouse-HRP, recombinant human interferon α-2b (rh-IFN-α-2b), recombinant human interferon α-8 (rh-IFN-α-8)

and leucocyte IFN-α were supplied by the Center for Genetic Engineering and Biotechnology (CIGB) in Havana, Cuba. The microtiter plates (96-well, Maxisorp), were from Nunc (Roskilde, Denmark). Proteinase K, trypsin, and pepsin were from Boehringer Mannheim (Germany). All other chemicals were of analytical grade and obtained from Sigma.

Methods

ELISA Procedure

Coating conditions: The effect of the temperature and time of incubation (37°C for 3 h or 4°C for 14–16 h) and the concentration of the Mab (0.5–40 μg/mL) in the coating were evaluated. The Mab diluted in carbonate 0.01 M/bicarbonate 0.03 M, pH = 9.6 buffer was added at the rate of 100 μL/well. After washing the plates three times with washing solution [(10 mM sodium phosphate and 145 mM NaCl (PBS), pH 7.2, containing 0.05% (v/v) Tween 20)], 80 μL/well of anti-mouse HRP-conjugated in 0.5% (w/v) BSA/Tween 20 0.05% (v/v)/PBS were added and the microplates incubated for 1 h at room temperature (RT, 25°C). The wells were washed 8 times with Tween 20 0.05% (v/v)/PBS and then 100 μL substrate solution was added (0.04% (w/v) *o*-phenylene diamine/citrate buffer, pH = 5.5, with 0.04% (v/v) H_2O_2). After 10–15 min incubation, 50 μL 2 M H_2SO_4 were added and the absorbance was read at 492 nm in a conventional plate reader.

Incubation of samples: The plates were coated for 3 h at 37°C and then three levels of temperature were assayed (4°C, 37°C and RT) in the incubation of the samples diluted in 0.5% (w/v) BSA/PBS or 0.05% (v/v) Tween 20/PBS. The samples were exposed during 1–6 h and the CB-IFNG.100 HRP-conjugated was used as detection antibody. The washing conditions, Mab HRP-conjugated incubation, and development reaction were the same as used in the above-mentioned coating conditions.

Validation of the Assay

Linearity/Range: The range of concentration values evaluated was from 400 to 5 ng/mL. Triplicate determination of each point was made. Calibration curves were obtained with different concentrations of the sample and the response measured in absorbance (492 nm). The least-squares method was applied to obtain the function describing a linear model. The determination coefficient (r^2) and probability associated with F Snedecor were determined

at different concentration ranges with Microsoft Excel software (Microsoft Corporation, USA). The accuracy and precision were calculated for each dilution. Working range was established between the highest and lowest concentration values with satisfactory accuracy and precision.

Limit of Detection (LOD) and Limit of Quantification (LOQ): LOD was the minimum concentration of a substance that generated a consistent response greater than the background of the test, and it was calculated as: (3 × standard deviation of the blank) × (concentration of sample)/OD of sample. LOQ was the smallest amount of an analyte that could be measured quantitatively in a sample, with acceptable accuracy and precision. We accepted the lowest value.

Bioassay

The biological activity was measured by inhibition of the cytopathic effect (CPE) in HEP-2 cells using the Mengo virus (ATCC No CCL23), as previously reported.[14] Cell monolayers in 96-well microtiter plates were incubated for 24 h at 37°C, under 3% CO_2 and 97% humidity, with IFN samples (serially diluted 1:2 in minimum essential medium with 2% fetal calf serum and 40 μg/mL gentamicin). Virus (10^7 TCID) was then added to each well and incubation proceeded under the same conditions until CPE (90% cell lysis) was evident (approx. 18–20 h) in the control virus wells (incubation without IFN). The degree of cell destruction was measured by fixing and staining the remaining cells with crystal violet. The cytophatic effect was determined using the plate photometer of an ultramicroanalytical system device (Tecnosuma, Havana, Cuba). A validated software (USD Pascal, CIGB) was used to convert the raw non-linear sigmoid data to regression through a probit transformation. The unit of antiviral activity is defined as the reciprocal of the sample dilution that yields a 50% protection from the virus CPE. The potency of each sample was expressed in IU compared to a secondary reference calibrated against international standard for IFN-γ (Gxg 01-902-535) (NIH, USA).

RESULTS AND DISCUSSION

Development and Validation of ELISA for rh-IFN-γ

During microplate coating, temperatures and times (3 h at 37°C or 14–16 h at 4°C) showed similar results. This allowed using one or another condition indistinctively. The optimal Mab-coat concentration proved

to be 10–15 μg/mL. To continue, 15 μg/mL was chosen to ensure the saturation of the microplate. The incubation of the samples at different times, for the three temperatures studied, showed a proportional increase of absorbance in time, while the antigen–antibody reaction augmented at low temperatures. These results can be caused by the instability of the IFN-γ molecule when it is exposed to high temperatures and extensive periods of incubation, a fact that has been evaluated by other researchers.[15] The best conditions for the incubation of the samples selected, guaranteeing an appropriate signal in the assay, were 2 h at 4°C in 0.05% (v/v) Tween 20/PBS (used for subsequent analyses), and the use of 0.5% (w/v) BSA/PBS as dilution buffer for the CB-IFNG.100 HRP-conjugated. The effect of powdered milk 0.05% (w/v)/PBS as sample buffer and blocking agent (1, 2, and 4% (w/v)/PBS) did not yield satisfactory results because of the high values of absorbance by non-specific bindings.

Linearity and range: As shown in Fig. 1A, the points obtained by plotting the absorbance values against concentration values appear as a sigmoid, and the "average curve" shown in Fig. 1B indicates a working range of 15–100 ng/mL (15, 20, 25, 50, 75, and 100 ng/mL) with absorbance values between 0.169 and 1.4 (background value = 0.069). The calculated F Snedecor associated probability was 0.07 and it was compatible with the data, at a determination coefficient above 0.98. The accuracy at each concentration (x) of the working range was analyzed using the Student-t test. The values of the experimental statistical Student-t (texp) calculated[16] were between 0.15 and 0.2. The accuracy was adequate, since the texp values were lower than the tabulated (4.302), in every concentration.

Precision: The precision of each point was calculated using the coefficient of variation (CV, %). It was below 15%.

Limits: The LOD and LOQ were 1.26 and 15 ng/mL, respectively.

Specificity: The immunoreactivity of different functionally related substances (rh-IFN-α-2b, rh-IFN-α-8, and Leucocyte IFN-α) under the assay conditions were measured and compared with that of rh-IFN-γ. Reaction was not observed within the range of the concentrations studied (data not shown). The absorbance signal obtained did not exceed the value of the corresponding non-specific absorptions. The ELISA is specific for rh-IFN-γ.

Characterization of the ELISA Assay

In order to evaluate how the immunoreactivity in the ELISA can be affected by denaturing conditions, and its relationship with the biological

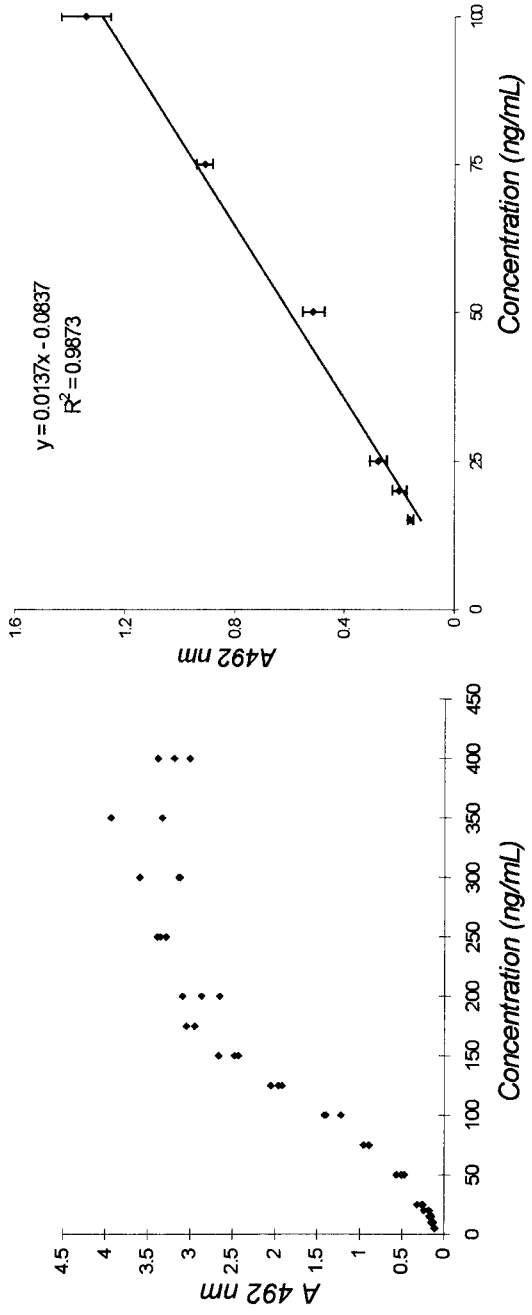

Figure 1. Standard curve for quantification of rh-IFN-γ by the ELISA assay. A: The standard Interferon Gamma was added at sandwich ELISA in different amounts (see abscissa, samples in triplicate). Points: plotting of concentration values (x) against absorbance values (492 nm) (y). B: The linear portion of the curve lies between 100 and 15 ng/mL. Data are the average of three experiments, each performed in triplicate. The bars indicate the standard error of independent determinations.

Table 1. Loss of antiviral and ELISA activity of rh-IFN-γ after treatment with different denaturing conditions.

	Residual activity (%)[a]	
Denaturing conditions	ELISA	Bioassay
None	100	100
SDS 0.001%[b]	66	64
SDS 0.01%[b]	0.23	0.247
Heat (37°C, 2 h)[c]	98	92
Heat (50°C, 2 h)[c]	46	39
Proteinase *K*[d]	Nd	Nd
Trypsin[d]	0.13	0.015
Pepsin[d]	24	18

[a]Residual activity (%) was defined as the relation between the treated and non-treated sample determined by both methods.
[b]Samples of purified rh-IFN-γ were incubated for 2 h at 4°C before testing.
[c]Samples of purified rh-IFN-γ were heated for 2 h at each temperature.
[d]A total of 50 μg of purified rh-IFN-γ was incubated for 2 h at 37°C in the presence of different proteases (1 μg) in a volume of 200 μL of PBS.
Nd: Not detected.

activity of the protein, different samples of rh-IFN-γ were incubated at 37 and 50°C, respectively. We also assayed the incubation of samples with different proteolytic enzymes (Proteinase K, Trypsin, and Pepsin) and samples at different concentrations of SDS (0.001 and 0.01% (w/v)). The samples were tested by ELISA and cytopathic inhibition bioassay. Table 1 shows a good correlation between the rh-IFN-γ levels after different denaturing conditions in both tests.

Keeping in mind the above results, we looked for a mathematical relationship between ELISA and bioassay. Samples at different steps of the production process of rh-IFN-γ in the range of $1–15 \times 10^6$ IU/mL were also evaluated by both ELISA and bioassay. The data obtained were processed by a regression and correlation analysis. The correlation coefficient obtained was 0.964 (Fig. 2). These results make this ELISA useful in estimating the biological activity.

Finally, we assayed the ability of this ELISA test to determine the optimal concentration of the surfactant Tween 80 for rh-IFN-γ stability during freeze-thawing period. Tween 80 was added in the concentration range of 0–1% (v/v) to two concentrations of rh-IFN-γ in PBS (40 and 400 μg/mL), and the samples were submitted to ten freeze-thawing cycles. As shown in Fig. 3, after final thawing without surfactant, only 3 and 29%

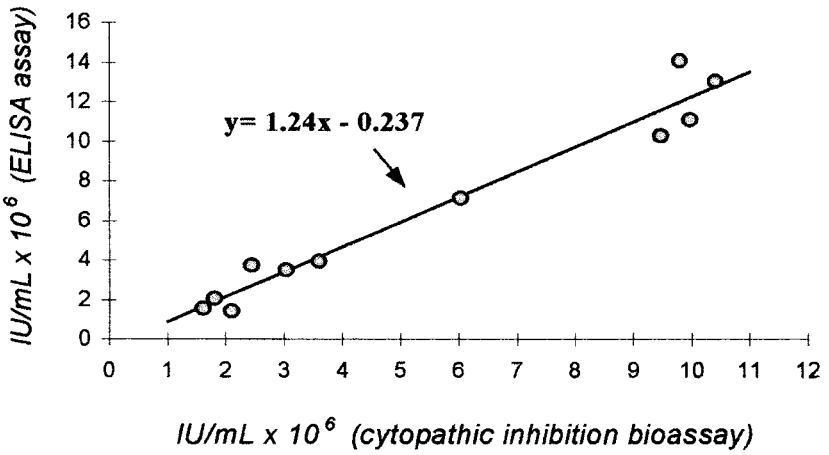

Figure 2. Correlation between ELISA (y) and bioassay (x) for samples of rh-IFN-γ.

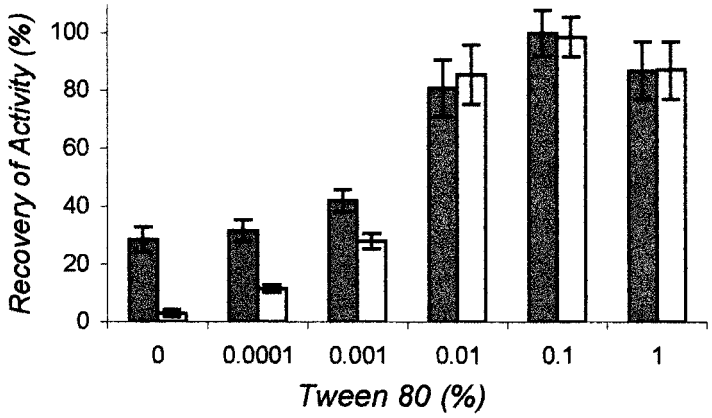

Figure 3. Recovery of activity of rh-IFN-γ in 0 to 1% of Tween 80 in PBS after ten freeze-thawing cycles. The recovery was calculated by ELISA in comparison to untreated control. 400 μg/mL of rh-IFN-γ. 40 μg/mL of rh-IFN-γ.

of the initial activity was recovered for the two concentrations assayed, respectively. Lower concentrations were more prone to lose activity during freeze-thawing, but 0.01–1% concentration of Tween 80 protected rh-IFN-γ during freeze-thawing, regardless of its concentration. The optimal concentration of Tween 80 was 0.1% in which the treated

sample retained almost 100% of activity, compared to the untreated control.

The ELISA assay described in this work has demonstrated the existence of a good correlation with bioassay, based on a single monoclonal antibody, and that it has certain advantages over bioassay, such as low cost, short assay completion time, high specificity (able to discriminate between different IFN types) and more resistance under the influence of environmental conditions. Therefore, this assay provides an excellent screening test for quantifying bioactive rh-IFN-γ in production processes and formulation studies.

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