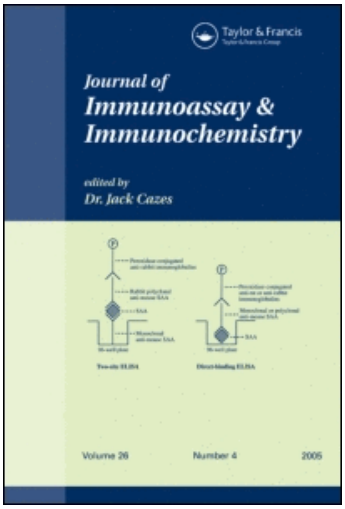

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HIGHLY SENSITIVE ENZYME IMMUNOASSAY OF HUMAN INTERFERON-β1

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ABSTRACT

A highly sensitive sandwich enzyme immunoassay for human interferon-β 1(HuIFN-β 1) was developed. HuIFN-β 1-containing samples and horseradish peroxidase(HRP)-labeled mouse anti-HuIFN-β1 monoclonal antibody(Fab') were incubated overnight at 2-10°C in the wells of a 96-well microtiter plate, onto which affinity-purified rabbit anti-HuIFN-β 1 polyclonal antibody was coated. The EIA was able to detect 0.5 IU/ml of HuIFN-β1, thus showing higher sensitivity than bioassay. The values obtained by the EIA closely paralleled those obtained by bioassay in the concentration which bioassay can detect. In order to detect the concentration below 0.5 IU/ml of HuIFN-β 1, the avidin/biotin-amplified EIA was also developed. The use of biotinylated mouse anti-HuIFN-β 1 monoclonal antibody ($F(ab')_2$) and HRP-avidin in the EIA made it possible to detect 0.1 IU/ml of HuIFN-β1. These EIAs were applied for the studies such as process control of HuIFN-β 1 production, pharmacokinetics of HuIFN-β 1, and determination of serum level of HuIFN-β1 in healthy subjects.

KEY WORDS: Human Interferon-β 1, Sandwich Enzyme Immunoassay, Monoclonal Antibody, Horseradish Peroxidase, Avidin/Biotin.

INTRODUCTION

Interferons represent a family of proteins, having not only antiviral activity but also other biological activities(1). On

the basis of their physicochemical and immunochemical properties, interferons can be distinguished into three major classes: IFN-α, -β, and -γ(2).

Human IFN-β1(HuIFN-β1) derived from human fibroblast cells has been used in clinical applications to treat cancers, hepatitis, and other diseases. After intravenous administration for clinical study, it is very important to monitor the amount of HuIFN-β1 in the circulation system. However, no assay method to detect extremely low concentrations of HuIFN-β 1 has been reported.

Although bioassay based on the inhibition of the cytopathic effect of viruses on human cells(3) is generally used, it is neither simple nor rapid. Moreover, bioassay can not discriminate IFN-types(HuIFN-α, -β, and -γ).

In order to detect concentrations below 5.0 IU/ml of HuIFN-β1, we attempted the development of highly sensitive enzyme immunoassays of HuIFN-β 1 using a mouse monoclonal antibody, YSB-1(4), and further their application on the determination of endogenous HuIFN-β1 in human serum.

MATERIALS AND METHODS

Interferons

HuIFN-β 1 was produced in the culture of human fibroblast cells super-induced(5) with poly(I):poly(C), cycloheximide, and actinomycin D, and purified by column chromatography(6). Recombinant HuIFN-β 1(rHuIFN-β 1) and mouse IFN-β (rMuIFN-β)

produced in E. coli were purified by the methods of Utsumi, et al.(7) and Matsuda, et al.(8), respectively. HuIFN-α and recombinant HuIFN-γ(rHuIFN-γ) were obtained from Red Cross Co. and Genentech Inc., respectively. The specific activities were $1.0x10^8$ IU/mg of HuIFN-β 1, $3.5x10^7$ U/mg of rMuIFN-β , $1.5x10^6$ IU/mg of HuIFN-α and $4.9x10^7$ U/mg of rHuIFN-γ .

Antibodies

Monoclonal antibody(YSB-1) in ascitic fluids was obtained from Yamasa Biochemicals Co. LTD, (Choshi, Japan)(4), was purified by ammonium sulfate precipitation (50% saturation). Polyclonal antibodies were prepared by immunization of rHuIFN-β 1 to rabbit as follows: subcutaneous injection of rHuIFN-β 1(500μg) with potassium alminum sulfate as an adjuvant was performed. After seven days from the 5th injection, the bleeding was carried out. The rabbit antisera were purified by ammonium sulfate precipitation (50% saturation) and futher by affinity chromatography on Affi-Gel 10(Bio-Rad) coupled with rHuIFN-β 1. The purified polyclonal antibodies were used as first antibody of EIA to be coated onto the wells of a 96-well microtiter plate.

Preparation of mouse monoclonal F(ab')2 and Fab'

Mouse anti-HuIFN-β 1 monoclonal antibody(200 mg) in 10 ml of 0.1M sodium acetate buffer, pH 4.5, containing 0.1M NaCl was digested to $F(ab')_2$ by incubation with 8 mg pepsin in 0.8 ml of the same buffer at 37°C for 20 hr. After adjusting pH to 8.0 with NaOH, the reaction mixture was passed through the column of

Sephacryl S-200 equilibrated with 0.1M borate buffer, pH 8.0, to isolate $F(ab')_2$. The $F(ab')_2$ fraction was applied onto an affinity column of Affi-Gel 10 coupled with rHuIFN-β 1. After washing with PBS, the elution was performed with 0.015 N HCl, and the pH was adjusted to 7.0 by adding 0.2 M phosphate buffer, pH 7.4. The affinity-purified $F(ab')_2$ dialysed against 0.1 M phosphate buffer, pH 6.0, containing 5 mM EDTA was reduced to Fab' by incubation with 0.1 M 2-mercaptoethylamine in 0.1 M phosphate buffer, pH 6.0, containing 5 mM EDTA at 37°C for 90 min, and applied onto a gel filtration column of Sephacryl S-200 equilibrated with 0.1 M phosphate buffer, pH 6.0, containing 5 mM EDTA to obtain Fab'.

Preparation of horseradish peroxidase(HRP)-Fab' and biotinylated $F(ab')_2$

Enzyme conjugate, HRP-Fab', was prepared by the method of Yoshitake, et al.(9). HRP(Grade I. Boehringer-Mannheim) was treated with N-(ε-maleimidocaproyloxy)succinimide to introduce maleimide groups. Then the maleimidated enzyme(1.0 mg) was allowed to react with thiol groups of the Fab'(1.2 mg) at 4°C for 20 hr and the conjugate formed was separated from unreacted components by gel filtration on Ultrogel AcA 44. The enzyme conjugate was kept at 4°C after adding 0.1% BSA and 0.005% sodium merthiolate as stabilizers.

Biotinylated $F(ab')_2$ was prepared by the method of Bergquist and Nilsson(10). The affinity-purified $F(ab')_2$(1.5 mg) was

dialysed against 0.1 M $NaHCO_3$ and concentrated to 1.0 ml by ultrafiltration. To the $F(ab')_2$ solution, 60 µl of N-hydroxysuccinimidobiotin(Pierce, Rockford, IL) (1 mg/ml) in dimethylsulfoxide was added and kept at room temperature for 4 hr. To remove excess N-hydroxysuccinimidobiotin, the reaction mixture was dialysed against PBS, and after adding BSA and sodium merthiolate, it was used for avidin/biotin-amplified EIA system.

EIAs for HuIFN-β1

Two EIA systems, sandwich EIA and avidin/biotin-amplified EIA, were developed. The former was performed as follows: affinity-purified rabbit anti-rHuIFN-β 1 in PBS (1 µg/ml, 100 µl/well) was coated onto the wells of the 96-well microtiter EIA plate (Nunc Immunoplate I) at 4°C overnight. After washing with PBS containing 0.05% Tween 20(washing buffer)(250 µl/well), the wells were blocked with 0.5% BSA in PBS containing 0.05% Tween 20 (250 µl/well) at room temperature for at least 1 hr. After washing once, 50 µl aliquiots of enzyme-Fab' conjugate and 200 µl of unknown HuIFN-β 1 samples or serial dilutions of HuIFN-β1 reference preparation were added to the wells. For dilution of the samples and reference preparation, 0.1 M phosphate buffer, pH 7.0, containing 0.1 % BSA and 0.05% Tween 20(assay buffer) was used. The plate was incubated at 4°C for 15-20 hr. After washing 3 times with washing buffer, 100 µl of enzyme substrate solution (McIlvaine buffer, pH 5.0, containing 0.08% o-phenylenediamine and 0.012% hydrogen peroxide) was added and

incubated in the dark at room temperature for 1 hr. Thereafter, the reaction was stopped by addition of 4.5 N H_2SO_4 (100 µl/well). Optical density of each well was measured by an immunoreader, Intermed NJ-2000, at 490 nm using 405 nm as reference. The amount of HuIFN-β 1 in samples was calculated from the standard curve of HuIFN-β 1 reference which had been standardized against the NIH reference for HuIFN- β1(G-023-902-527, NIH, Bethesda, MD). The assay was usually performed in triplicate.

On the other hand, avidin/biotin-amplified EIA was performed using the same microtiter plate coated with rabbit anti-rHuIFN-β1. After the wells were blocked with 0.5% BSA in PBS containing 0.05% Tween 20, 50 µl aliqiots of biotinylated $F(ab')_2$ and 100 µl of unknown HuIFN- β1 samples or serial dilutions of HuIFN- β1 reference preparation were added. The plate was incubated at 4°C for 15-20 hr. After washing 3 times, 100 µl of 1:10,000 dilutions of HRP-avidin D(Vector Lab. Inc., CA) was added to each well and the plate was allowed to stand at room temperature for 2 hr. After washing 3 times, the enzyme substrate (100 µl/well) was added to the wells and incubated at room temperature for 1 hr. The degree of enzyme reaction was determined by the same method as described above.

The determination of serum samples was performed by two methods. One was direct determination of 2 fold-diluted serum sample and the other was as follows: HuIFN- β 1 reference preparations in concentrations of 0, 5, 10, and 20 IU/ml were

added to serum sample. The serum samples having four different HuIFN- β1 concentrations were determined by the above EIAs and the four values were plotted against concentration of the added HuIFN- β1, and then the endogenous HuIFN- β1 level was extrapolated.

Interferon Assay

A cytopathic inhibition effect assay in human FL cells with vesicular stomatitis virus or Sindbis virus as a challenge virus was used for HuIFN- β1 titration(3). A laboratory standard which was standardized against the international reference for HuIFN-β1 (G-023-902-527) was included in all bioassays.

RESULTS AND DISCUSSION

Detection Limit of HuIFN-β1

The standard curve of sandwich EIA was shown in Fig.1. The detection limit of HuIFN- β1 was 0.5 IU/ml using 200 μl of samples. This value shows that the EIA has a higher sensitivity than bioassay whose detection limit is around 5 IU/ml(3). Futhermore, the detection limit of HuIFN- β1 by immunoassay which has ever been reported is around 30 IU/ml (11). The detection limit of our sandwich EIA was found to depend on the volume of IFN-sample (Curves A and B in Fig.1), so 200 μl of samples was used in the assay to obtain the higher sensitivity. The sandwich EIA detected both natural and recombinant HuIFN-β1s but not HuIFN-α, rHuIFN-γ and rMuIFN-β (data not shown), thus also showing a high specificity.

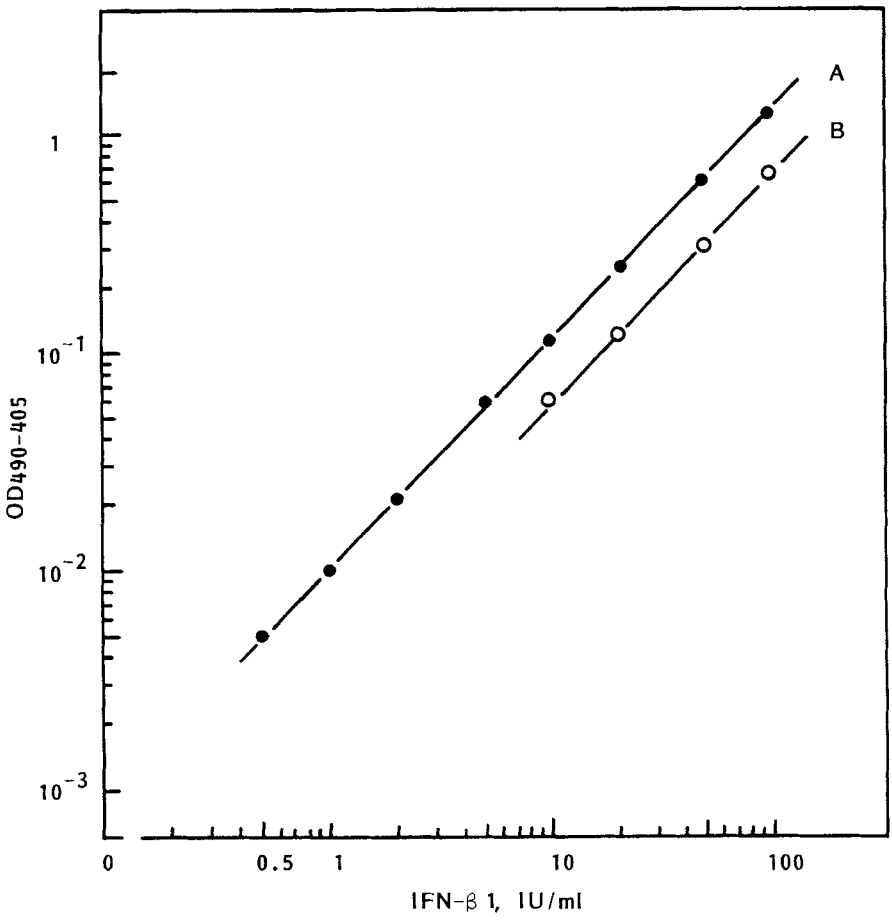

FIGURE 1 Standard curves of sandwich EIA for HuIFN- β1 using 200 μl(A) and 100 μl(B) of HuIFN- β1 reference dilutions.

In order to detect the concentration below 0.5 IU/ml of HuIFN- β1, the avidin/biotin-amplified EIA was also developed. As shown in Fig.2, the EIA was able to detect 0.1 IU/ml of HuIFN-β1 using 100 μl of samples. The detection limit corresponds to 1.1 amol/well, calculated by taking the specific activity and

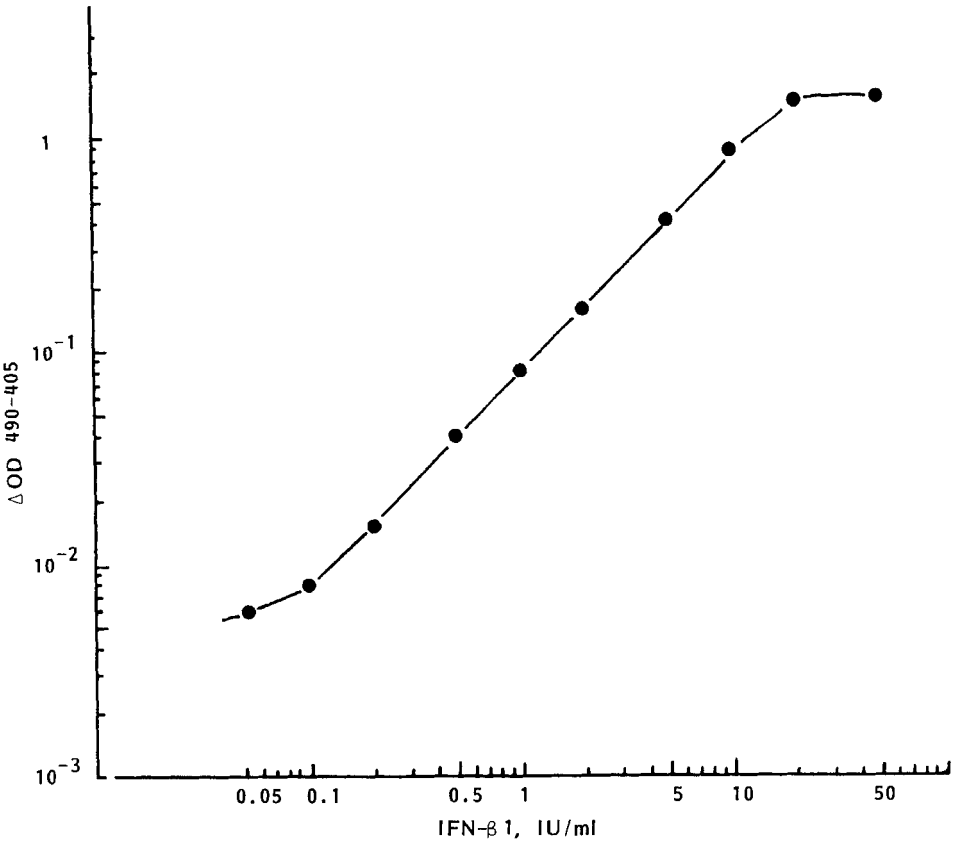

FIGURE 2 Standard curve of avidin/biotin-amplified EIA for HuIFN- β1 using 100 µl of reference dilutions. Background was 0.020.

molecular weight of HuIFN- β1 to be $4x10^8$ IU/mg (12) and 23,000(7), respectively. Unlike the sandwich EIA, the detection limit of the avidin/biotin-amplified EIA was not improved by increasing sample volume. Therefore, 100 µl of samples was used in the amplified EIA. The background of the amplified EIA was 0.011-0.026, slightly higher than that of the sandwich EIA, 0-0.006.

TABLE 1

Determination of heat-treated samples by sandwich EIA and bioassay

Incubation Time	Sandwich EIA *	Bioassay
0 min	1.2×10^6 U/ml(100 %)	1.1×10^6 U/ml(100 %)
2	1.1×10^6 (92)	1.1×10^6 (100)
10	6.2×10^5 (52)	6.9×10^5 (63)
30	1.7×10^4 (1.4)	1.2×10^4 (1.1)
60	6.5×10^3 (0.5)	2.8×10^3 (0.3)

* rHuIFN- β1 reference for bioassay was used as EIA standard.

Comparison with Bioassay

In order to examine whether the sandwich EIA detects inactivated HuIFN- β1 molecules as well as the active molecules, rHuIFN- β1 in a medium, 20 mM phosphate buffer containing 150 mM NaCl and 40% ethyleneglycol(pH 7.4), was incubated at 60 °C. After 0, 2, 10, 30 and 60 min, each aliquot was taken out, diluted 10 times with minimum essential medium (MEM) containing 5% fetal calf serum (FCS) and frozen to stop the further inactivation. As shown in Table 1, the residual activities determined by both the sandwich EIA and bioassay were nearly identical, indicating that the EIA detected only biologically active HuIFN- β1 molecules.

After intravenous administration of HuIFN- β1 to dogs for the pharmacokinetic study, HuIFN- β1 titers in the sera at various intervals were determined by the sandwich EIA, and the values were well correlated to those by bioassay using the same

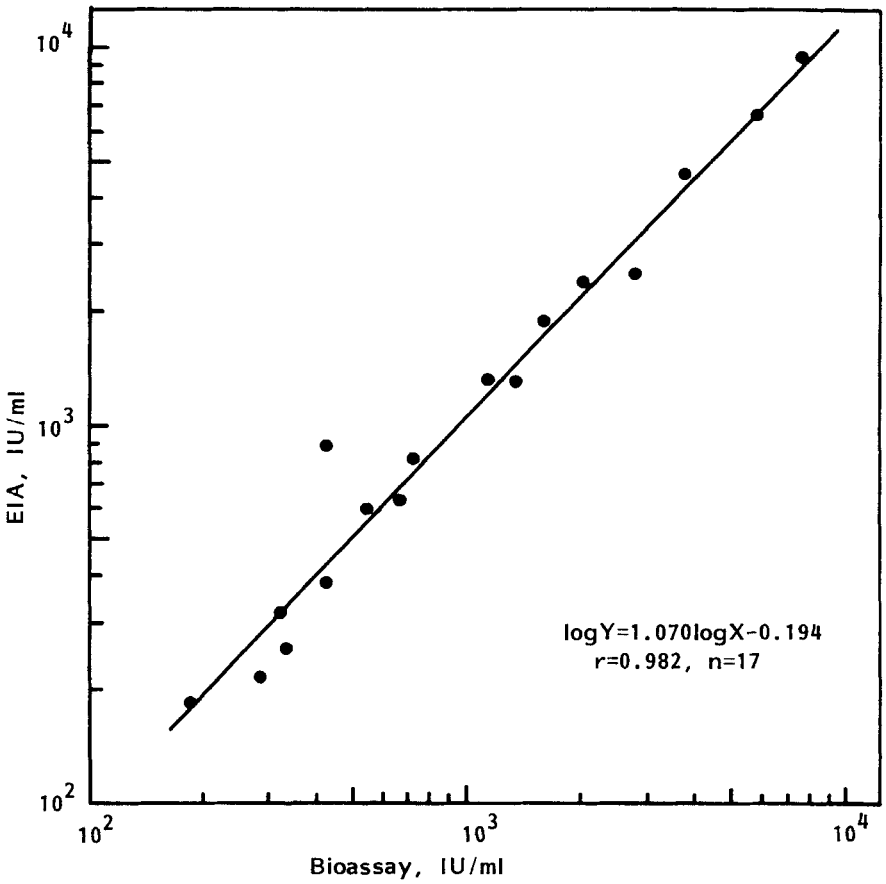

FIGURE 3 Correlation between HuIFN- β1 titers obtained by sandwich EIA and bioassay.

laboratory reference which had been standardized against the NIH reference(Fig.3).

Assay Variations

In the sandwich EIA, at five different HuIFN- β1 samples over the range of 10-200 IU/ml, coefficients of intra- and inter-

assay variations were 2.0-9.7 % (n=12), and 2.3-4.9 % (n=4), respectively. The obtained CV(%) was in normally much below 10% in the range of 10-200 IU/ml, while occasionally greater than 10% in the range of 0.5-10 IU/ml.

In the avidin/biotin-amplified EIA, coefficients of intra- and inter-assay variations were normally less than those in the sandwich EIA. However, in the case of serum samples containing very low concentration of HuIFN- β1, CV(%) values were occasionally higher than the normal values, probably due to the interference of unknown non-HuIFN-β1 substances which arose only in the amplified EIA.

Advantages of the present EIAs

As mentioned above, the present EIAs could detect very low concentrations of HuIFN- β 1. This might be led by an establishment of the suitable assay condition. As for the antigen-antibody reaction time and its temperature, 18 hr-incubation at 2-10°C was much better than 30 min-incubation at room temperature(Fig.4). HuIFN- β1 in low concentrations is easily denatured even at room temperature(13). Therefore, the reaction of HuIFN- β1 with the specific antibodies in immunoassay should be performed at the temperature below 10°C. Futhermore, the prolonged reaction time may lead to the denature of antigen. In the present EIAs which detect only biologically active HuIFN-β1, 15-20 hr-incubation at 2-10°C was the most suitable condition for the reaction of HuIFN- β1 with the antibodies.

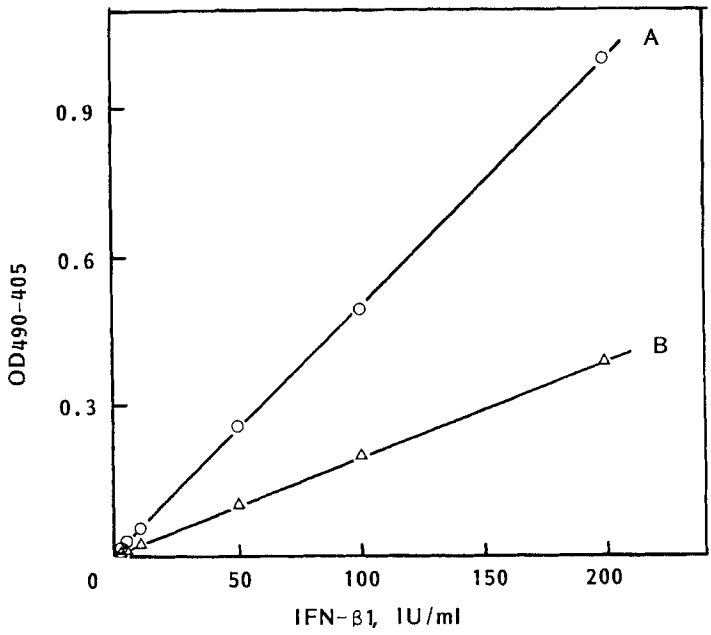

FIGURE 4 Comparison of reaction condition for sandwich EIA: (A) 18 hr-incubation at 4°C, and (B) 30 min-incubation at room temperature. In the assay, 100 µl of HuIFN-β1 reference dilutions was used.

Application

The sandwich EIA can detect HuIFN- β1 in various media such as animal serum(or plasma) and MEM containing 5% FCS. From the above results of no medium-interference and the high specificity for biologically active HuIFN- β1, the sandwich EIA could be applied for the studies such as process control of HuIFN- β1 production, pharmacokinetics of HuIFN- β1 (14), and the determination of serum level of HuIFN- β1 in healthy subjects and patients.

TABLE 2

Serum level of HuIFN-β1 in healthy subjects determined by sandwich EIA, avidin/biotin-amplified EIA (A/B EIA) and bioassay

Serum No.	Age	Sex	HuIFN- β1 (IU/ml serum)						
			Sandwich EIA			A/B EIA		Bioassay	
			A	B	(r)	C	(r)	D	E
1	39	M	5.6	7.4	(0.997)	6.5	(0.999)	12.9	<4.7
2	30	M	<2.0	0.5	(0.997)	2.5	(0.997)	<4.7	
3	25	M	<2.0	0.9	(0.999)	149	(0.245)	<4.7	
4	29	M	3.4	5.6	(0.982)	5.3	(0.999)	<4.7	
5	28	F	<2.0	0.7	(0.998)	19.2	(0.998)	<4.7	
6	35	M	<2.0	1.0	(0.999)	2.9	(0.985)	<4.7	
7	24	M	<2.0	0.7	(0.999)	2.3	(0.992)	<4.7	
8	29	M	<2.0	0.9	(0.999)	1.0	(0.994)	<4.7	
9	28	M	<2.0	1.5	(0.997)	3.8	(0.984)	<4.7	
10	26	M	<2.0	1.2	(0.999)	2.2	(0.990)	<4.7	
Average 2.04±2.40									

A; Direct determination with 2 fold-diluted serum. In this case, the detection limit was 1.0 IU/ml.

B and C; The values were obtained by extrapolation. The detail was described in the text. The values in parentheses mean coefficient of correlation, r, between the determination values and the concentrations of HuIFN-β1 added to serum sample.

D and E; Bioassay using FL cell line and Sindbis virus.

E; HuIFN- β1 titer after the antibody treatment. The treatment was performed by incubation of the serum with rabbit anti-HuIFN- β1 antibody at room temperature for 30 min.

As a preliminary study, the determination of serum level of HuIFN- β1 in healthy subjects was performed by the sandwich EIA, the amplified EIA and bioassay. As shown in Table 2, several methods were attempted to detect endogenous HuIFN-β1 in human serum. Using 200 μl of 2 fold-diluted serum samples, only two of ten donors' sera could be detected by the sandwich EIA (Column A

in Table 2). The serum level of HuIFN- β1 was also obtained by extrapolation on the basis of the determination values of HuIFN-β1-added serum sample. By the method, the sandwich EIA could clearly distinguish the two sera having high values from the other sera (Column B in Table 2), while the amplified EIA could not (Column C in Table 2). Discrepancy in serum No.3, 5 and 9 was appeared, especially huge in serum No.3. Owing to the fact that serum No.3 showed an abnormal value in the coefficient of correlation (r), the unbelievable value was obtained by extrapolation. Besides 10 serum samples in Table 2, HuIFN- β1 levels in other 20 serum samples were determined by the EIAs (data not shown). The amplified EIA showed higher values in almost all of the samples than the sandwich EIA. The higher values by the amplified EIA may be due to serum-interferance with avidin and/or biotin in very low concentrations of HuIFN- β1. Therefore, the amplified EIA may not be suitable for detection of HuIFN- β1 in serum. On the other hand, bioassay could detect only one subject's serum level, which was neutralized with rabbit anti-rHuIFN- β1 antibody (Columns D and E in Table 2). It is not clear why No.1 subject had a higher serum level than any other subjects. Serum level of HuIFN- β1 in various kinds of patients will be studied in relation to the diseases.

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