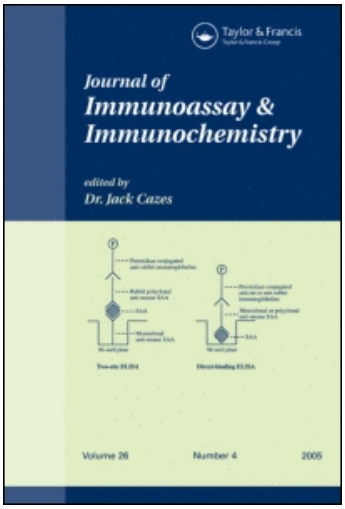

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N. Moriya[a]; K. Kato[a]; N. Suzuki[b]; T. Yamada[a]; O. Nishimura[a]
[a] Biotechnology Laboratories, Central Research Division, Takeda Chemical Industries, Ltd., Osaka, Japan [b] Chemistry Laboratories, Central Research Division, Takeda Chemical Industries, Ltd., Osaka, Japan

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AN ENZYME-IMMUNOASSAY FOR HUMAN INTERLEUKIN-2

N. Moriya, K. Kato, N. Suzuki,† T. Yamada
and O. Nishimura

Biotechnology Laboratories and †Chemistry Laboratories,
Central Research Division, Takeda Chemical Industries, Ltd.,
Yodogawa-ku, Osaka 532, Japan

ABSTRACT

A highly sensitive enzyme-immunoassay (EIA) for human interleukin-2 (IL-2) has been established. The assay is based on a sandwich method that uses two kinds of anti-IL-2 antibodies raised against *Escherichia coli*-derived recombinant IL-2 (rIL-2). An affinity-purified-anti-IL-2 goat IgG was used as the first antibody and the Fab' fragment of an affinity-purified-anti-IL-2 rabbit IgG was used as the second antibody after being coupled with horseradish peroxidase (HRP). As little as 30 pg/ml of IL-2 was detected by the EIA, indicating that this method was about 100 times more sensitive than the bioassay using an IL-2-dependent murine natural killer cell line, NKC3. There was a good correlation between the EIA and the bioassay ($r = 0.998$).

KEY WORDS: Human Interleukin-2 (IL-2), Enzyme-immunoassay (EIA), Pharmacokinetics

Proofs to Norihiko Moriya

Biotechnology Laboratories, Central Research Division, Takeda Chemical Industries, Ltd., Yodogawa-ku, Osaka 532, Japan

INTRODUCTION

Interleukin-2 (IL-2) is a lymphokine produced by T-cells when they are activated with either an antigen or a mitogen.[1-3] It regulates the immune response, and the growth and differentiation of T-cells. In addition, recent reports[4-6] indicate that IL-2 may be of potential use for treating neoplastic diseases or AIDS. The gene coding for human IL-2 was cloned from a Jurkat cell line and sequenced by Taniguchi *et al.*[7] We also succeeded in cloning the cDNA from activated human peripheral blood leukocytes and its expression in *Escherichia coli*.[8] In the previous papers,[9,10] we described the purification and characterization of recombinant human interleukin-2 (rIL-2) produced in *Escherichia coli*.

To clinically evaluate this rIL-2, a more sensitive assay system than the bioassay[11] is needed to determine the serum concentration of rIL-2. A study on determining IL-2 level by EIA has been reported.[12] However, this method is not sensitive enough to examine the IL-2 level in clinical samples. Here we report the establishment of a highly sensitive EIA system for IL-2.

MATERIALS AND METHODS

Materials

rIL-2 used throughout these laboratories was prepared by a recombinant DNA technique as described previously.[9,10] Recombinant human interferon-α (rIFN-α) and interferon-γ (rIFN-γ) were prepared according to the method described previously.[13,14] Recombinant human epidermal growth factor (r human EGF) was purchased from Wakunaga

Pharmaceutical Co., Ltd. (Osaka, Japan). Mouse EGF and bovine insulin were from Toyobo Co., Ltd. (Osaka, Japan) and Sigma Chemical Co. (Missouri, U.S.A.), respectively. Other materials were obtained from commercial sources and used without further purification: bovine serum albumin (Daiichi Kagaku Co., Tokyo, Japan), new born calf serum (M. A. Bioproducts, Maryland, U.S.A.), HRP-conjugated anti-rabbit IgG (Miles-Yeda, Illinois, U.S.A.), HRP-conjugated anti-goat IgG (Cappel, Pennsylvania, U.S.A.), 30% hydrogen peroxide (Wako Pure Chemicals, Osaka, Japan), and *o*-phenylenediamine (Wako Pure Chemicals).

Preparation of rIL-2-conjugated Sepharose 4B

rIL-2-conjugated Sepharose 4B for affinity chromatography was prepared according to the manufacturer's manual (Pharmacia Fine Chemicals, Uppsala, Sweden) using BrCN-activated Sepharose 4B (1 g) and rIL-2 (11.95 mg). The coupling yield was 98.0%.

Preparation of anti-IL-2 rabbit serum and anti-IL-2 goat serum

A rabbit (2 kg, JW-NIBS) was immunized with rIL-2 (1.2 mg) in complete Freund's adjuvant injected at multiple subcutaneous sites along the dorsal surface and intramuscularly into both thighs. After 2, 4, and 6 weeks, the animal was given the same dose of rIL-2 in complete Freund's adjuvant. One week after the final booster injection, blood was taken from the central ear vein, allowed to clot at room temperature for 3 hr and at 4°C for 16 hr, and then centrifuged (2250 x g, 10 min). The supernatant was used as anti-IL-2 rabbit serum. The anti-serum titer was around $10^6 \sim 10^7$ when assayed by EIA using HRP-conjugated anti-rabbit IgG.

An anti-IL-2 goat serum was prepared in the same way. The antiserum titer was around $10^6 \sim 10^7$ when assaayed by EIA using HRP-conjugated anti-goat IgG.

Preparation of anti-IL-2 rabbit IgG and anti-IL-2 goat IgG by affinity chromatography

An IgG fraction of anti-IL-2 rabbit serum (20 ml) was prepared by ammonium sulfate precipitation. After dialysis against 0.02 M borate buffer (pH 8.0), the IgG fraction was chromatographed on a column of rIL-2-coupled Sepharose 4B (1.2 x 4.0 cm). The specific antibody fraction was eluted with 0.2 M glycine-HCl (pH 2.0) and the pH of the eluate was immediately adjusted to 7.0 with 1 M Na_2HPO_4 and 1 N NaOH. After dialysis against 0.02 M borate buffer (pH 8.0), the affinity-purified IgG fraction was stored at 4°C until used.

The affinity-purified anti-IL-2 goat IgG was prepared in essentially the same manner.

Preparation of HRP-conjugated anti-IL-2 rabbit Fab'

The HRP-conjugated anti-IL-2 rabbit Fab' was prepared in the same manner as that described by Imagawa, *et al.*[15] The affinity-purified anti-IL-2 rabbit IgG (6.44 mg) was incubated with pepsin (120 μg, Sigma Chemical Co., Missouri, U.S.A.) at 37°C and at pH 4.5 for 16 hr in a final volume of 1.5 ml. After the reaction mixture was incubated, its pH was adjusted to 8.0 with 1 N NaOH and $F(ab')_2$ was separated from other components on a Sephadex G-150 column (1.5 x 45.0 cm, Pharmacia, Uppsala, Sweden). The column was developed with 0.01 M borate buffer (pH 8.0). The $F(ab')_2$ was concentrated in a Collodion-Bag (Sartorius GmbH, Göttingen, West Germany), dialyzed against 0.1 M acetate buffer

(pH 5.0), and reduced with 20 mM 2-mercaptoethylamine. The Fab' fragment was separated from lower molecular weight components on a Sephadex G-25 column. The column was developed with 0.1 M phosphate buffer (pH 6.0) containing 5 mM EDTA, and the desired fractions were pooled.

HRP (10 mg, Boehringer Mannheim, Mannheim, West Germany) disssolved in 1.4 ml of 0.1 M phosphate buffer (pH 6.8) was mixed with N-(4-carboxycyclohexylmethyl)maleimide-N-hydroxysuccinimide ester (10 mg, Zieben Chemicals, Tokyo, Japan) dissolved in 100 µl of N,N-dimethylformamide. The mixture was stirred at room temperature for 1 hr and then centrifuged (1600 x g, 10 min). The supernatant was directly applied to a Sephadex G-25 column (1.0 x 80.0 cm). The column was developed with 0.1 M phosphate buffer (pH 6.8). The desired fractions were pooled and stored at 4°C.

The maleimide-treated HRP thus obtained was coupled with the Fab' fragment at 4°C for 20 hr. The reaction mixture was centrifuged, and the supernatant fluid was applied to an Ultrogel AcA 44 column (1.0 x 80.0 cm, LKB, Bromma, Sweden). The column was developed with 0.1 M phosphate buffer (pH 6.8). The desired fractions were pooled and stored at 4°C in the presence of 0.1% bovine serum albumin and 0.001% thimerosal. The molar ratio of Fab' fragment and HRP in this conjugate was calculated from its absorbance, to be approximately 1.7.

EIA procedures

Nunc-immunoplate (A/C Nunc, Roskilde, Denmark) was used. One hundred microliters of the affinity purified anti-IL-2 goat IgG solution (15 µg/ml) was added to each well. The plate was incubated for 16-20 hr at

4°C and then rinsed three times with 300 µl of 0.1 M phosphate buffer (pH 6.8) containing 0.15 M NaCl (PBS). Then, 200 µl of 0.1% bovine serum albumin solution was placed in each well. The plate was incubated at 4°C for 16-20 hr, and rinsed three times with PBS. One hundred microliters of a test sample (an IL-2 solution or a test serum diluted with PBS containing 10% fetal calf serum) was put in each well. The plate was incubated at 4°C for 16-20 hr and then washed well with PBS. Next, 100 µl of a HRP-conjugated anti-IL-2 rabbit Fab' solution (5 µg/ml) was added to each well and the plate was incubated at room temperature for 4 hr and then washed three times with PBS. One hundred microliters of the substrate solution (40 mM *o*-phenylenediamine in 0.1 M citrate buffer (pH 5.5) containing 0.066% hydrogen peroxide plus thimerosal) was added to each well and the plate was incubated for 20-40 min in the dark. One hundred microliters of 2 M H_2SO_4 was added to stop the reaction and the absorbance at 492 nm was read in Titertek Multiskan (Flow Labs, California, U.S.A.).

Bioassay of IL-2

Fifty microliters of 8 x 10^5 NKC3 cells/ml were added to each well containing the serial 2-fold dilutions (50 µl) of IL-2 standard and test samples. The cells were cultured at 37°C for 24 hr in a 5% CO_2 incubator and then 20 µl of 5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-diphenyl-tetrazolium bromide solution were added to each well. After culturing for another 4.5 hr, 100 µl of sodium dodecyl sulfate-0.01N HCl solution was added to each well and then the micro-culture plate was re-incubated overnight. Absorbance at 590 nm was measured by a Titertek Multiskan.[11]

RESULTS AND DISCUSSION

To enhance the sensitivity and specificity of the EIA, we used the affinity-purified anti-IL-2 goat IgG and the HRP-conjugated rabbit Fab' derived from the affinity-purified anti-IL-2 rabbit IgG as the first and the second antibodies, respectively.[15] As little as 30 pg/ml of rIL-2 was detectable by this EIA method (Fig. 1). The sensitivity was defined as the least amount of rIL-2 that could be distinguished from zero within 99% confidence limits. The EIA is approximately 100 times more sensitive than the bioassay using an IL-2 dependent murine natural killer cell line (NKC3), in which the lower detection limit of rIL-2 was approximately 3 ng/ml.[11] An EIA procedure reported by Robb[12] has the same magnitude of sensitivity as the bioassay.

Natural IL-2 (nIL-2) derived from human peripheral blood leukocytes[16] gave the same calibration curve as that for rIL-2 in our EIA system (Fig. 1). These results indicate that the antibodies in this sandwich EIA system recognize the same peptide epitopes of rIL-2 and nIL-2 molecules because the rIL-2 preparation used in this study was completely non-glycosylated.

The EIA method was highly specific to IL-2 since rIFN-α, rIFN-γ, r human EGF, mouse EGF and bovine insulin gave less than 0.0001% reactivity on molar basis compared with rIL-2 (TABLE 1). It is worth noting that reduced rIL-2 gave only 11.2% reactivity and that the same preparation retained 8.9% of the activity of intact rIL-2 molecule. The EIA method seems to only detect biologically active molecules. Within- and between-assay precision at various rIL-2 concentrations are shown in TABLE 2. Good recoveries (98.6-104.0%) were obtained for three samples

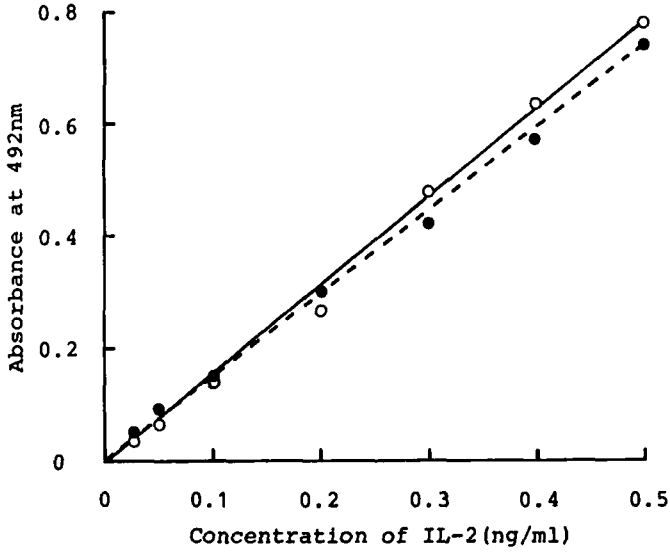

FIGURE 1. Calibration curve for rIL-2 and nIL-2. Microplate wells were coated with 15 μg/ml purified anti-IL-2 goat IgG. The coated wells were then incubated for 4 hr at room temperature with 100 μl of each of the indicated concentrations of rIL-2 or nIL-2, followed by 100 μl anti-IL-2 rabbit Fab'-HRP at a concentration of 1:50 dilution. After the substrate solution was added, incubation was carried out for 40 min. —o—, rIL-2; ---●---, nIL-2. Each point represents the mean of three determinations.

TABLE 1

Cross Reactivity of Related and Unrelated Polypeptides[a)]

Polypeptide	Relative reactivity (%)
rIL-2	100.0
Reduced rIL-2[b)]	11.2
rIFN-α	< 0.0001
rIFN-γ	< 0.0001
rHuman EGF	< 0.0001
Mouse EGF	< 0.0001
Bovine insulin	< 0.0001

a) Polypeptide samples (100, 10, 1 and 0.1 μg/ml) were subjected to the EIA. The reactivity was calculated based on the absorbance and molar concentration of each polypeptide.

b) A rIL-2 solution (1.0 mg/ml) was reduced with 200 mM dithiothreitol at pH 5.0 and 25°C for 16 hr.

TABLE 2

Within- and Between-Assay Variations[a)]

Sample	rIL-2 added (ng/ml)	n	rIL-2 measured (ng/ml)	Recovery (%)	CV[b)] (%)
Within-assay	1.0	10	1.04 ± 0.11[c)]	104.0	10.6
	3.0	10	3.05 ± 0.15	101.7	4.9
	10.0	10	9.95 ± 0.37	99.5	3.7
Between-assay	1.0	5	1.03 ± 0.05	103.0	4.9
	3.0	5	3.05 ± 0.05	101.7	1.6
	10.0	5	9.86 ± 0.21	98.6	2.1

a) Ten determinations each for three different rIL-2 concentrations were carried out for assessing within-assay variation. Five determinations each on successive five days for three different rIL-2 concentrations were carried out for assessing between-assay variation.

b) % Coefficient of variation.

c) Mean ± S.D.

of rIL-2 with coefficient of variations less than 10.6% and 4.9% for within-assay and between-assay, respectively.

To determine if this EIA method was of practical use, we used it to measure exogeneously added rIL-2 in the serum. Blood was taken from three healthy volunteers and the sera were obtained, in which nIL-2 level was below the detection limit of this EIA system. The test specimens were prepared by adding various amounts of rIL-2 to the sera. After the samples were left at room temperature for 1 hr, the concentrations of rIL-2 in the sera were determined. The recovery was good indicating that this

TABLE 3

Recovery of rIL-2 Added to Serum Samples[a)]

Human serum	rIL-2 added (ng/ml)	rIL-2 measured (ng/ml)	Recovery (%)
A	0	0	
	0.5	0.40	80.0
	1	1.10	110.0
	2	2.20	110.0
	4	3.85	96.3
	8	7.45	93.1
			97.9 ± 12.6[b)]
B	0	0	
	0.5	0.60	120.0
	1	1.25	125.0
	2	2.00	100.0
	4	3.85	96.3
	8	7.50	93.8
			107.0 ± 14.4
C	0	0	
	0.5	0.55	110.0
	1	1.15	115.0
	2	1.95	97.5
	4	3.85	96.3
	8	7.85	98.1
			103.4 ± 8.5

a) rIL-2 (0.5~8 ng/ml) was added to three normal human sera. After incubation for 1 hr at room temperature, the concentration of rIL-2 in the samples was determined by EIA. Each value was obtained by duplicate determinations.

b) Mean ± S.D.

procedure can be used to measure rIL-2 in the serum (TABLE 3). This EIA system is proved to be more advantageous than bioassay methods, because it is not influenced by inhibitor(s) in the serum.[17)]

To examine the correlation between the EIA and bioassay methods, test specimens containing different amounts of rIL-2 were prepared and

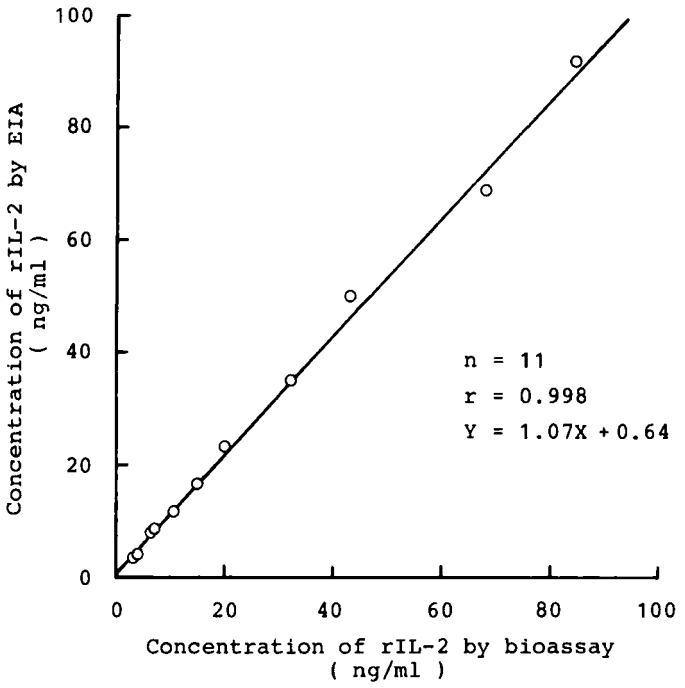

FIGURE 2. Correlation between the values obtained by EIA and those obtained by bioassay for rIL-2. Each point represents the mean of three determinations.

the concentrations of rIL-2 were measured by both methods. The result (Fig. 2) indicates that there is a good correlation between the two procedures ($r = 0.998$). Therefore, the values obtained by this EIA method are judged to be associated with biological activity.

These experimental results have indicated that this newly developed EIA system is expected to be of practical use and may be useful to measure rIL-2 in clinical samples. In fact, it has been partially proved to be suitable for surveying rIL-2 levels in patients during

pharmacokinetics studies. Details of these studies will be described elsewhere.

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