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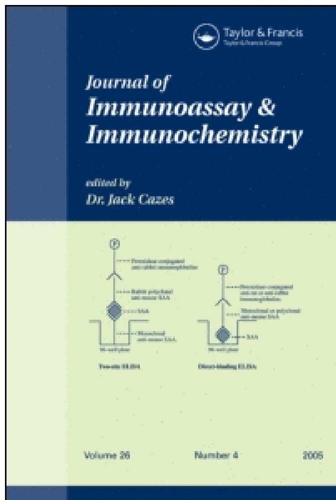

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An Enzyme Immunoassay for Human Lymphotoxin

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AN ENZYME IMMUNOASSAY FOR HUMAN LYMPHOTOXIN

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ABSTRACT

A highly sensitive and specific enzyme immunoassay (EIA) for human lymphotoxin (hLT) has been developed. The assay is based upon a sandwich system employing two kinds of anti-hLT antibodies with neutralizing activity. One of them was mouse monoclonal antibody raised against Escherichia coli-derived recombinant hLT with a deletion of 20 amino-terminal amino acids and used as labelled antibody. The other was rabbit antibody raised against the carboxyl-terminal portion of hLT and used as solid-phase antibody.

The EIA employing such a combination was able to detect less than 50 pg/ml of hLT, showing that this method was approximately 5-10 times higher sensitivity than the conventional bioassay employing L929 cell-lysis. The mean recovery of hLT added to serum specimens was 101% and the coefficients of variation were 3.3-7.8% (intra-assay) and 2.9-17.2% (inter-assay). There was a good correlation between the present EIA and the bioassay (r=0.93).

(KEY WORDS: Human Lymphotoxin (hLT), Enzyme Immunoassay (EIA), Carboxyl-Terminal Peptide of hLT, Recombinant hLT Muteins with a Deletion of Amino-Terminal Amino Acids)

INTRODUCTION

Lymphotoxin (LT) is a lymphokine produced by activated lymphocytes. It was first characterized as a glycoprotein which has anti-proliferative

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activity on neoplastic cell lines but not on primary cell cultures and normal cell lines (1,2). In 1984 Gray et al. reported the construction and expression in Escherichia coli (E. coli) of a DNA encoding human LT (hLT) and some of the in vitro and in vivo biological activities of the recombinant LT (3). Recently LT has been applied to a clinical field as well as tumor necrosis factor (TNF) and the assays are required to determine the concentrations of LT in sera and tissues. An L929 cell-lytic bioassay can be used for that purpose (4), but the assay is time-consuming and cannot distinguish LT from other cytotoxic substances like TNF. Bringman and Aggarwal have developed a specific enzyme immunoassay (EIA) which was about as sensitive as the standard L929 bioassay (5).

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The present study also describes a sensitive and specific EIA for determining the LT levels in sera, plasma specimens, culture supernatants and E. coli extracts. In the study rabbit antibody against the carboxyl-terminal (C-terminal) portion of hLT and mouse monoclonal neutralizing antibody were used as solid-phase and labelled antibodies, respectively.

MATERIALS AND METHODS

Recombinant hLT muteins

Complementary DNA (cDNA) encoding hLT was isolated from a human peripheral blood cDNA library using the oligonucleotide probes (6) and the plasmids for expression of hLT-cDNA in E. coli were constructed using ptrp 781 vector with the trp promotor, operator and trp leader ribosome binding site (7,8). The cDNA genes for LT and its muteins, which lack the first 4, 9

and 20 amino acids at the N-terminus, were obtained by standard procedures including digestion with restriction enzymes, separation and ligation of DNA fragments (8), as described by Maniatis et al. (9).

E. coli strain DH-1 transformed with various plasmids using the $CaCl_2$-RbCl procedure (9) was grown in M9 medium containing 5 μg/ml tetracycline or 20 μg/ml ampicillin. After incubation at 37°C for 4 hours, 25 μg/ml of indole acrylic acid was added, followed by further 4 hour-cultivation at 37°C. The cells were collected and lysed by incubation at 5°C for one hour with 0.2 mg/ml lysozyme and sonication on ice for one minute. After centrifugation at 12,000 rpm for 20 minutes, the resulting supernatants were applied to bioassays and EIAs, or to further purification procedures.

An hLT mutein with a deletion of 20 N-terminal amino acids was purified by salting-out E. coli extracts with saturated 40% ammonium sulfate and then by sequential chromatographies employing DEAE-Sepharose CL-6B and Blue-Sepharose CL-6B columns (8). The purified hLT mutein was emulsified in Freund's complete adjuvant (FCA) and injected subcutaneously into mice at the amount of 20 μg per mouse.

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Bioassay for hLT activity

Cytotoxic activity of hLT was determined by a method developed by Spofford et al. (4) with a slight modification. Briefly, two-fold serial dilutions of test samples were prepared in 96-well plastic tissue culture plates (Nunc, Denmark) and to each well L929 cells were added at a density of 2×10^3 cells/well in RPMI 1640 medium containing 5% fetal calf serum (FCS) and mitomycin C (0.5 μg/ml). After incubation at 37°C for 3 days in a humidified CO_2 incubator, the number of viable cells was measured by a

colorimetric method using 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (10). The LT unit per ml (U/ml) represents the reciprocal of a sample dilution which causes 50% cytotoxicity under the assay conditions.

As for a neutralization test, two-fold serial dilutions of antibody samples were added to an equal volume of 20 U/ml hLT solution and incubated at 37°C for one hour, followed by the L929 cytotoxicity assay described above.

Antibody production

Antibodies against the C-terminal portion of hLT were prepared by immunizing rabbits with chemically-synthesized peptide conjugated with bovine serum albumin (BSA). In brief, C-terminal LT fragment peptide (152-171) synthesized by a conventional solid-phase method was mixed with BSA and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (Wako Pure Chemicals, Japan) in molar ratios of 8:1:100, and incubated overnight at 5°C. The conjugates were emulsified with an equal volume of FCA and then injected at multiple subcutaneous sites along the dorsal surface. Three antisera showed high affinity to hLT with neutralizing activity and one of them was used as solid-phase antibody for EIA after purified by affinity chromatography using an hLT-bound cellulofine column.

Monoclonal antibodies (MAbs) were obtained from hybridomas produced by fusing spleen cells of a BALB/c mouse immunized against a recombinant LT mutein, which lacks the first 20 amino acids at the N-terminus (8), with P3-X63-Ag8-U1 mouse myeloma (11). Antibody levels in hybridoma culture media were determined by the EIA employing hLT-coated microplates (Nunc) and horse-radish peroxidase (HRPO)-labelled anti-mouse

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immunoglobulin. Four MAbs were obtained and two of them showed neutralizing activity to hLT. The neutralizing one selected for this study was IgG_{2b} isotype and purified from an ascites fluid by a conventional method employing ammonium sulfate precipitation and protein A-column chromatography (12). The IgG_{2b} MAb was biotinylated with N-hydroxy-succinimidobiotin (Pierce Chem. Co., Ill) (13) and used as labelled antibody for the present method.

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Enzyme immunoassay procedures

A 96-well microplate was coated with 100 μl per well of 0.1 M phosphate buffer (PB; pH 8.0) containing 1 μg of rabbit antibody against the C-terminal portion of hLT. After incubation overnight at RT, 100 μl of a blocking solution containing 2% BSA was added and allowed to stand at 5°C until used.

The antibody-coated plates were washed with 0.02 M PB (pH 7.3) containing 0.15 M NaCl and 0.1% Tween 20 (PBS-T), and then 100 μl of LT-containing sample solutions diluted with PBS containing 1% BSA was added to each well, followed by incubation at RT for two hours. After washing with PBS-T, 100 μl of biotinylated MAb was added and incubated at RT for two hours. After additional washing with PBS-T avidin-HRPO conjugate (Pierce Chem. Co.) was added and the plates were incubated at RT for another one hour, followed by the enzyme reaction.

As substrates were used 6 mM hydrogen peroxide and 40 mM o-phenylene diamine in 0.1 M citrate buffer (pH 5.5) and the plates were incubated at RT for 20 minutes until addition of 2 N H_2SO_4 (14). Optical density of each

well was measured at 492 nm by using a Titertek Multiskan (Flow Laboratories Inc., Virg.).

RESULTS

Antibodies against hLT

Three rabbits were immunized with C-terminal fragment peptide of hLT (152-171) and all of them produced anti-hLT antibodies with both high affinity and neutralizing activity. One of them was purified by salting-out with ammonium sulfate and by subsequent affinity chromatography using an hLT-bound column, then used as solid-phase antibody. Such purified antibody gave two to five times sensitive EIA than crude antibody preparations obtained only by salting-out or DEAE-cellulose column chromatography procedures.

Mouse MAb preparations purified by a protein A-column were biotinylated and the labelled antibody was used in combination with rabbit antibody-coated microplates. Figure 1 depicts the dose-response curves for hLT in the sandwich EIA, showing no crossreaction with hTNF. The sensitivity for hLT was 0.02 U/well and 0.2 U/ml, which means the present assay could detect less than 50 pg/ml of hLT.

Assay variations

Coefficients of variation (CV) in the EIA were assessed at five different hLT levels in sera and *E. coli* extracts. The results for intra-assay variations (n=8) were 0.93±0.07 U/ml (CV=7.5%), 1.96±0.09 U/ml (CV=4.6%), 2.15±0.10 U/ml (CV=4.7%), 3.90±0.13 U/ml (CV=3.3%) and 4.62±0.36 U/ml

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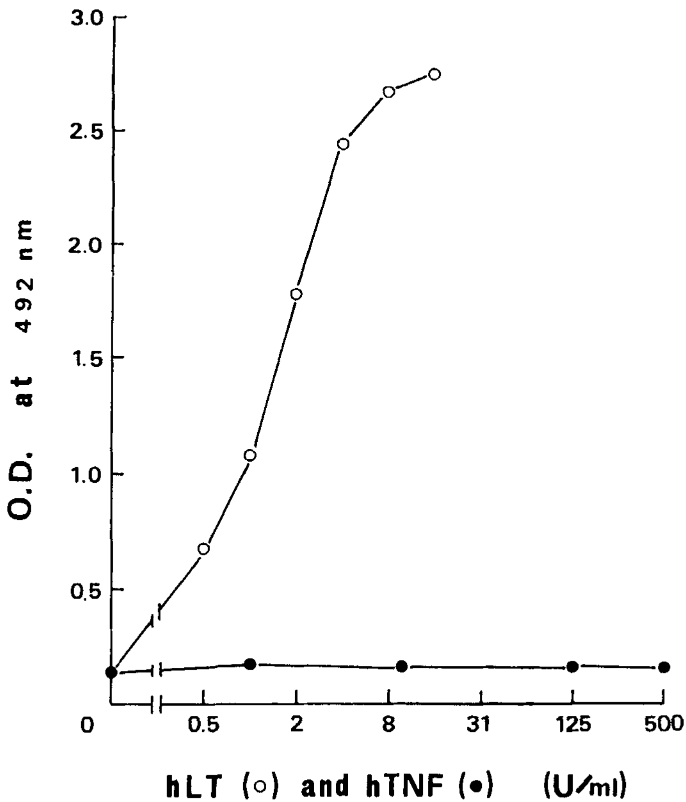

FIGURE 1
Dose-response curves for human lymphotoxin (hLT) and tumor necrosis factor (TNF) in the enzyme immunoassay.

(CV=7.8%). The results for inter-assay variations (n=5) were 0.70±0.02 U/ml (CV=2.9%), 1.64±0.21 U/ml (CV=12.8%), 1.91±0.12 U/ml (CV=6.3%), 3.66±0.27 U/ml (CV=7.4%) and 3.89±0.67 U/ml (CV=17.2%). The present method gave low variations in all the samples tested, indicating good reproducibility and preciseness.

TABLE 1

Recovery of hLT Added to Human Sera[a)]

Serum no.	Added (U/ml)	Measured (U/ml)	Recovered (%)
1	0.50	0.40	80
	1.00	0.90	90
	2.00	1.97	99
	4.00	3.87	97
	8.00	7.12	89
2	0.50	0.48	96
	1.00	1.15	115
	2.00	2.38	119
	4.00	4.80	120
	8.00	7.37	92
3	0.50	0.45	90
	1.00	1.05	105
	2.00	2.23	112
	4.00	4.64	116
	8.00	7.87	98

a) Mean ± S.D. = 101 ± 12 %

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Recovery of hLT added to sera

Various amounts of hLT were added to three human sera to give final concentrations of 0.5-8.0 U/ml and the mixtures were assayed for hLT by this method. The recovery of hLT added ranged from 80 to 120% with an average of approximately 101% for 15 determinations (TABLE 1).

Plasma levels of hLT in rats

Purified hLT preparations were intravenously injected into five male Spraque-Dawley rats (220-250 g) at dose of 10,000 units per rat. Plasma

TABLE 2

Plasma hLT Levels Determined by EIA after A Single Intravenous Injection of 10,000 Units of hLT per Rat

Rat no.	Time after intravenous injection			
	20 minutes (U/ml)	One hour (U/ml)	Two hours (U/ml)	Four hours (U/ml)
1	36.4	19.2	10.4	<1.0
2	51.2	18.0	5.2	<1.0
3	51.2	20.8	3.2	<1.0
4	96.8	27.6	13.6	<1.0
5	59.2	27.6	5.2	<1.0
Mean ± S.D.	59.0±20.3	22.6±4.1	7.5±3.9	<1.0

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specimens were taken 20 minutes to 6 hours after injection and assayed for hLT by the present EIA. Plasma hLT levels rapidly declined and became undetectable by 4 hours after administration (TABLE 2).

Comparison of EIA and bioassay

The concentration of hLT in 23 samples including rat plasma specimens, culture supernatants from human T cells transformed by a retrovirus (15) and *E. coli* extracts was determined by both EIA and bioassay employing L929 cell-lysis. There was a good correlation (r=0.93) between hLT values determined by the two methods (Fig. 2), and the regression line determined by the method of least-squares was $y = 0.92x + 4.2$. The results indicate the EIA values are associated with biological activity.

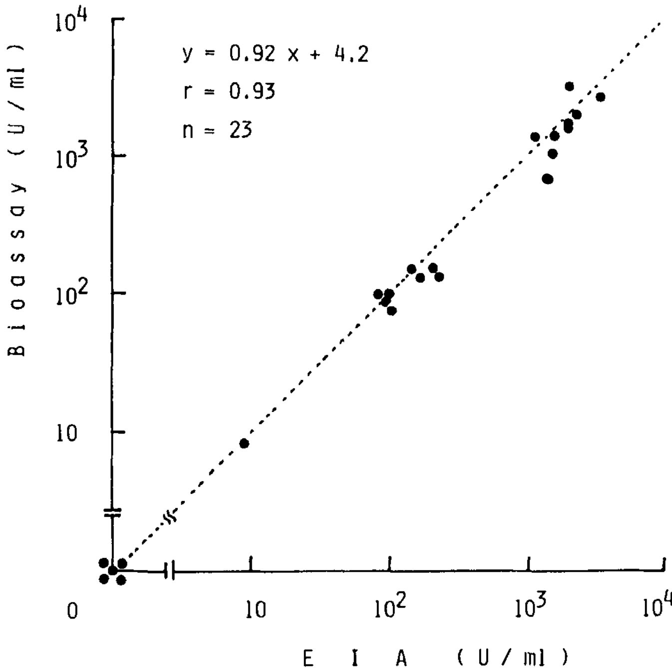

FIGURE 2
Correlation between human lymphotoxin levels measured by the enzyme immunoassay (EIA) and bioassay employing L929 cell-lysis.

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DISCUSSION

Rabbit antisera against C-terminal fragment peptide of hLT (152-171) were all reactive to an hLT whole molecule and also capable of neutralizing its cytotoxic activity. These results correlated well with the lack of cytotoxic activity in a truncated LT polypeptide lacking its last 16 amino acids (3) and with the distinct homology in the hydrophobic C-termini of hLT and hTNF which have the shared cytotoxic activities (16,17). All the antisera obtained here, however, were not able to crossreact with TNF, as

described by Bringman and Aggarwal (5), showing that hLT and hTNF are antigenically distinct molecules although they interact with a common receptor (16).

Four mouse MAbs were obtained by using a recombinant hLT mutein (21-171) as immunogen and two of them had neutralizing activity to hLT. One of two neutralizing antibodies was used as labelled antibody in combination with solid-phase rabbit antibody in the present EIA system. Another system was also performed, where rabbit antibody was used as labelled antibody and mouse MAb as solid-phase antibody, but the system resulted in five to ten times lower sensitivity than the present system. Thus the present EIA can detect as little as approximately 0.02 unit and the sensitivity was 5 to 10 times higher than that of the EIA reported by Bringman and Aggarwal (5).

The present EIA was compared with the conventional L929 cell-lytic bioassay, and the EIA values were found to be associated with biological activity. Moreover, biologically-active hLT muteins lacking the first 4, 9 and 20 amino acids and natural hLT preparations obtained from human T cell transformed by a retrovirus gave similar dose-response curves to that given by a recombinant hLT whole molecule (1-171). These results suggested the present method might detect only bioactive hLT molecules and the probable reason was that both of the two antibodies used here were neutralizing antibodies which recognized active sites on an hLT molecule.

The present EIA using such a sensitive and specific combination of two neutralizing antibodies was also performed reproducibly and precisely, and therefore it will aid in elucidating the role of hLT in normal and disease states and in diagnosing patients given hLT.

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