A sandwich-enzyme immunoassay for the quantification of lipoprotein lipase and hepatic triglyceride lipase in human postheparin plasma using monoclonal antibodies to the corresponding enzymes

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Abstract We have developed a sandwich-enzyme immunoassay (EIA) for the quantification of lipoprotein lipase (LPL) and hepatic triglyceride lipase (HTGL) in human postheparin plasma (PHP) using monoclonal antibodies (MAbs) directed against the corresponding enzymes purified from human PHP. The sandwich-EIA for LPL was performed by using the combination of two distinct types of anti-LPL MAbs that recognize different epitopes on the LPL molecule. The immunoreactive mass of LPL was specifically measured using a β-galactosidase-labeled anti-LPL MAb as an enzyme-linked MAb, an anti-LPL MAb linked with the bacterial cell wall as an insolubilized MAb, and purified human PHP-LPL as a standard. The sandwich-EIA for HTGL was carried out by using two distinct anti-HTGL MAbs that recognize different epitopes on HTGL. The limit of detection was 20 ng/ml for LPL and 60 ng/ml for HTGL. Each method yielded a coefficient of variation of less than 6% in intra- and inter-assays, and a high concentration of triglyceride did not interfere with the assays. The average recovery of purified human PHP-LPL and -HTGL added to human PHP samples was 98.8% and 97.5%, respectively. The immunoreactive masses of LPL and HTGL in PHP samples, obtained at a heparin dose of 30 IU/kg, from 34 normolipidemic and 20 hypertriglyceridemic subjects were quantified by the sandwich-EIA. To assess the reliability of the measured mass values, they were compared with the corresponding enzyme activities measured by selective immunoinactivation assay using rabbit anti-human PHP-LPL and -HTGL polyclonal antisera. Both assay methods yielded a highly significant correlation in either normolipidemic (r = 0.945 for LPL; r = 0.932 for HTGL) or hypertriglyceridemic subjects (r = 0.989 for LPL; r = 0.954 for HTGL). The normal mean (±SD) level of lipoprotein lipase mass and activity in postheparin plasma was 223 ± 66 ng/ml and 10.1 ± 2.9 μmol/h per ml, and that of hepatic triglyceride lipase mass and activity was 1456 ± 469 ng/ml and 26.4 ± 8.7 μmol/h per ml, respectively. The present sandwich-enzyme immunoassay methods make it possible to study the molecular nature of LPL and HTGL in PHP from patients with either primary or secondary hyperlipoproteinemia. —**Ikeda, Y., A. Takagi, Y. Ohkaru, K. Nogi, T. Iwanaga, S. Kurooka, and A. Yamamoto.** A sandwich-enzyme immunoassay for the quantification of lipoprotein lipase and hepatic triglyceride lipase in human postheparin plasma using monoclonal antibodies to the corresponding enzymes. *J. Lipid Res.* 1990. **31:** 1911–1924.

Supplementary key words hyperlipoproteinemia • lipoprotein metabolism

Plasma triglycerides are mainly transported in the form of chylomicrons and very low density lipoproteins (VLDL). These two classes of triglyceride-rich lipoproteins are catabolized by lipoprotein lipase (LPL) at the first step in their metabolism (1–4), and their catabolic products appear to be further processed by hepatic triglyceride lipase (HTGL) (3, 5). Both enzymes are normally anchored to capillary endothelium in a catalytically active form and are released into circulation after intravenous injection of heparin (6). Recently, both enzymes were purified from human postheparin plasma (PHP) and characterized (7,

Abbreviations: EIA, enzyme immunoassay; LPL, lipoprotein lipase; HTGL, hepatic triglyceride lipase; PHP, postheparin plasma; MAb, monoclonal antibody; SD, standard deviation; VLDL, very low density lipoprotein; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EDTA, ethylenediamine tetraacetic acid; SIIA, selective immunoinactivation assay.

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8). The purified human LPL and HTGL give subunit molecular weights of 61,000 and 65,000, respectively (7, 8), and they are catalytically active in a monomeric form (8).

In biochemical studies on patients with hyperlipoproteinemia, the analysis of LPL and HTGL in PHP samples is usually performed as an important diagnostic measure to estimate whether or not their plasma lipoprotein disorders result from a primary defect of LPL or HTGL. Previous studies have been focused on selectively measuring LPL and HTGL activities in PHP samples obtained after injection of heparin at a dose ranging from 10 IU/kg to 100 IU/kg (6, 9, 10). Clinically, the level of LPL activity in PHP inversely correlates with the concentration of triglycerides in plasma and the primary deficiency in its activity results in Type I hyperlipoproteinemia with severe fasting chylomicronemia (11). In the case of secondary hyperlipoproteinemia with diabetes mellitus or a nephrotic syndrome, LPL activity in PHP is also known to be reduced (12). HTGL activity in PHP decreases as a result of some liver diseases, hypothyroidism, and a genetic disorder of this enzyme (3); in some cases, an accumulation of β-VLDL is reported. In order to further investigate the functional abnormalities of LPL and HTGL enzymes in genetic disorders of each enzyme or in secondary hyperlipoproteinemia, selective measurement of LPL and HTGL immunoreactive masses as well as of activities in human PHP is important. There have been two reports about immunoassays that were applicable to LPL in human PHP with an enzyme-linked immunosorbent assay (ELISA) using a single monoclonal antibody (MAb) directed against partially purified human milk LPL (13) or against bovine milk LPL (14). For the purpose of developing a sensitive and specific method to determine LPL mass in human PHP, a specific antibody raised against LPL purified from human PHP and the pure PHP-LPL as a standard are required, but such an assay method has not yet been reported. No paper has been published dealing with an assay method for the determination of the immunoreactive mass of HTGL in human PHP.

In this study, we report a sandwich-enzyme immunoassay (EIA) for the quantification of LPL mass in human PHP samples using the combination of two distinct anti-human PHP-LPL MAbs that recognize different epitopes on the LPL molecule, and are directed against LPL purified from human PHP. A sandwich-EIA for HTGL using two distinct anti-human PHP-HTGL MAbs that recognize different epitopes on HTGL protein is also reported. Our data indicate that the present sandwich-EIA methods for LPL and HTGL can be practically applied to the study of enzyme abnormalities in PHP samples from either primary or secondary hypertriglyceridemic subjects.

MATERIALS AND METHODS

Subjects and PHP samples

Thirty four healthy subjects (28 males and 6 females) aged 23–58 years with a mean ± SD of 37.68 ± 8.95 years were recruited as a control group. Their total triglyceride and cholesterol concentrations were 85.9 ± 33.9 mg/dl and 191.9 ± 30.9 mg/dl (mean ± SD), respectively.

The hypertriglyceridemic subjects (15 males and 5 females aged 22–61 years with a mean ± SD of 48.3 ± 10.1 years) were referred to our National Cardiovascular Center. Their total triglyceride and cholesterol concentrations were 946.9 ± 717.7 mg/dl and 243.4 ± 99.7 mg/dl (mean ± SD), respectively. They had functionally active apoC-II and no plasma inhibitors against LPL.

After the subjects fasted overnight, blood samples were collected in tubes (Venoject, VT-070NA, TERUMO, Japan) containing Na_2-EDTA (1 mg/ml) before and 10 min after the injection of heparin (Novo Industry, A/S, Denmark) at a dose of 30 IU/kg of body weight, unless other wise mentioned. The tubes were immediately placed in an ice bath. Plasma was separated by centrifugation at 1500 *g* for 10 min at 4°C and stored at −80°C until used.

Standard human PHP-LPL and human PHP-HTGL

LPL and HTGL were purified from human PHP with some modifications according to the methods described in our previous paper (8). After the final purification step, an aliquot of the LPL or HTGL preparation was used for protein analysis and the remaining preparations were immediately mixed with bovine serum albumin (BSA) at a final concentration of 5% to minimize the adsorption to the inside of the test tubes and the inactivation of the corresponding enzymes. The purified human PHP-LPL and -HTGL were used as a standard protein to establish the sandwich-EIA for LPL and HTGL. The purified enzymes each gave a single protein band on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (8). The specific activities of the purified LPL and HTGL were estimated to be 44.8 ± 1.9 mmol/h per mg (mean ± SD, n = 3) and 18.1 ± 0.78 mmol of free fatty acid released/h per mg (mean ± SD, n = 3), respectively, using tri[9,10-^{3}H]olein-gum arabic emulsion as a substrate. The specific activities of LPL and HTGL used in this experiment were slightly higher than those of the previously isolated LPL (33.95 ± 5.25 mmol/h per mg, n = 5) and HTGL (15.97 ± 1.55 mmol/h per mg, n = 5) (8). The protein concentration of the purified human PHP-LPL and -HTGL was determined by Micro BCA protein assay (Pierce). BSA was used as a standard.

Preparation of MAbs to human PHP-LPL and PHP-HTGL

The purified PHP-LPL (30 μg of protein) emulsified with complete Freund's adjuvant (DIFCO Lab., Detroit, MI) was subcutaneously injected in the back or abdomen of BALB/c mice (4 weeks female, Shizuoka Experimental Animal Center, Japan) three times at intervals of 20 days. Four days after the last immunization, spleen cells (2.2×10^8) were prepared from the mouse and fused to myeloma cells (P3 × 63-AG8-658, 7×10^7) in the presence of 33% (w/w) polyethylene glycol 1540 (15). After 1 min incubation, the cell suspension was diluted with 20 ml of serum-free RPMI-1640 medium and the cells were collected by centrifugation. The cell pellet was suspended in a HAT medium and the suspension (200 μl each) was distributed among the 96 wells of a micro-titer plate (Flow Laboratories Inc.). The plates were incubated at 37°C in a 5% CO_2 atmosphere, and fresh HAT medium was added to each well every 4 days. Hybridoma, producing anti-human PHP-LPL antibody, were selected by testing the hybridoma culture medium by a dot immunobinding assay using pure PHP-LPL (37 ng of protein) fixed to a HA-cellulose membrane in a Millipore millititer plate (Millipore Corp., Bedford, MA). The antibody-antigen complexes were visualized by a Vectastain ABC kit (Pk-4002, Vector Lab. Inc., Burlingame, CA) according to the supplier's directions. The positive hybridoma (134 wells/1920 wells) were subcloned by the method of limiting dilution (0.3 cell/well). The subcloned hybridoma were screened by testing both the dot immunobinding assay and the immunoprecipitation of the purified PHP-LPL catalytic activity. Five hybridoma showed a strong positive reaction with LPL fixed on the HA-cellulose membrane and were capable of immunoprecipitating LPL catalytic activity. These anti-human PHP-LPL MAbs were designated: 1(1)D2B2, 1(7)D9B5, 1(9)B4B4, 2(4)D2G8, and 2(10)F8F9. The MAbs were further enriched in ascites by intraperitoneally injecting the hybridoma (2×10^6) into BALB/c mice treated with Pristane. Immunoglobulins in ascites were purified by ammonium sulfate fractionation and DEAE-cellulose chromatography. The subclasses of these MAbs were determined by a Mouse Monoclonal Typing Kit (ICN ImmunoBiologicals, Lisle, IL). Anti-LPL MAb-1(7)D9B5 was IgG2b, and the others were all IgG1.

MAbs against human PHP-HTGL were prepared using the purified PHP-HTGL (30 μg of protein/mouse) and the same procedures described above. Hybridoma, producing PHP-HTGL antibodies, were obtained from two different experiments. In the first screening with the dot immunoassay using pure PHP-HTGL (70 ng of protein/well), positive hybridoma were found in 34 wells/295 wells in the first experiment and in 54 wells/158 wells in the second experiment. The positive hybridoma were further subcloned. Eight of them reacted strongly positive with HTGL fixed on the HA-cellulose membrane and were capable of immunoprecipitating the purified human PHP-HTGL catalytic activity. These anti-human PHP-HTGL MAbs were designated: 1(1)A7A6, 1(2)E12E7, 1(6)A5B3, 1(11)A3H3, 1(11)C1B8, 1(11)D10D9, 2(4)F12C12, and 2(9)A9A1. The MAbs were further enriched in ascites of BALB/c mice. Immunoglobulin was purified as described above. The subclass of the MAbs was all IgG1.

Quantification of IgG1 from the native MAbs was done by a Mouse IgG1 RID kit (The Binding Site Ltd., Birmingham, UK). The amount of IgG1 was expressed as μg/ml.

Labeling of MAbs with β-galactosidase

The coupling of anti-human PHP-LPL and -HTGL MAbs with *Escherichia coli* β-galactosidase (Boehringer-Mannheim, FRG) was performed using methods described previously (16). The purified MAbs (400 μg of protein/400 μl of 0.1 M phosphate buffer, pH 7.0) were mixed with 150 μl of 0.2% of *m*-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) in dioxane and stirred for 30 min at 25°C. Then, 10 ml of 0.1 M phosphate buffer (pH 7.0) was added to the above reaction mixture and stirred for 10 min at 25°C. The reaction mixture was treated two times with 10 ml of the same phosphate buffer using PM 30 membrane (Amicon) to remove unreacted MBS. The resulting MBS-MAbs solution (1.5 ml) was mixed with β-galactosidase (250 μg of protein) dissolved in 200 μl of 50% ammonium sulfate solution and the mixture was stirred for 2 h at 25°C and overnight at 4°C. The reaction mixture (1.7 ml) was applied to a Sepharose 6B column (1.5 × 100 cm) equilibrated with buffer A (0.04 M phosphate buffer, pH 7.0, containing 0.15 M NaCl, 0.1% BSA, and 0.1% NaN_3). The column was eluted with buffer A at a flow rate of 0.24 ml/min. β-Galactosidase activity in each eluate (3 ml each) was measured with 2-nitrophenol-β-D-galactoside as a substrate. The eluates (50 ml) containing MAbs coupled with β-galactosidase were pooled and diluted 50- to 100-fold with buffer A when used for the EIA assay.

Quantitation of IgG1 in the MAbs labeled with β-galactosidase was done by a competitive binding-EIA using standard mouse IgG1, rabbit anti-mouse IgG1 antibody, alkaline phosphatase-labeled mouse IgG as an enzyme-labeled antigen, and goat anti-rabbit IgG antibody linked to the bacterial (*Lactobacillus plantarum*) cell wall according to the methods described previously (16). The amount of IgG1 was expressed as μg/ml.

Linking of MAbs to the bacterial cell wall

The anti-human PHP-LPL and -HTGL MAbs were linked to the bacterial cell walls using the methods described previously (16). The cell-wall suspension (0.4 g

in 50 ml of distilled water) was mixed with MAbs (5 mg of protein each) dissolved in 1 M sodium acetate buffer (pH 4.0) containing 1-ethyl-3(3-dimethylaminopropyl)carbodiimide (EDC) and glutaraldehyde. The mixture was stirred for 1 h at 25°C and then centrifuged for 10 min at 2000 *g* to sediment the MAbs linked to the cell walls. The pellet was washed three times with buffer A by repeating resuspension and centrifugation. The insolubilized MAbs were finally suspended in buffer A containing 5% BSA.

Quantification of IgG1 in the MAbs linked to the cell walls was done by a competitive binding-EIA using standard mouse IgG1, rabbit anti-mouse IgG1 antibody, β-galactosidase-labeled mouse IgG as an enzyme-labeled antigen, and goat anti-rabbit IgG antibody linked to the cell walls (16). The amount of IgG1 was expressed as μg/ml.

Grouping test of MAbs

The grouping test of the obtained anti-human PHP-LPL MAbs was performed by checking which combinations of two MAbs could sandwich pure human PHP-LPL as an antigen. A β-galactosidase-labeled anti-LPL MAb and an anti-LPL MAb linked with the cell wall were mixed with the purified PHP-LPL dissolved in buffer A, and the mixture was incubated for 15 min at 37°C. Then, the mixture was centrifuged at 2000 *g* for 10 min. The resulting pellet was suspended in 500 μl of buffer A and the β-galactosidase activity was measured as described above. When two MAbs recognize different epitopes on LPL protein, β-galactosidase activity is able to be detected in the ternary complexes consisting of β-galactosidase-labeled MAb, LPL, and MAb linked with the cell wall. In contrast, no β-galactosidase activity is detected when two MAbs recognize the same epitope on LPL, because only binary complexes consisting of MAb linked with cell wall and LPL are precipitated. Under these experimental procedures, five anti-human PHP-LPL MAbs and eight anti-human PHP-HTGL MAbs were tested. In the case of anti-LPL MAbs, 1(1)D2B2, 1(7)D9B5, and 2(4)D2G8 were of the same group, while they were distinct from both 1(9)B4B4 and 2(10)F8F9. Thus, 1(1)D2B2 and 2(10)F8F9 were selected to establish a sandwich-EIA for LPL. For the sandwich-EIA for HTGL, 2(4)F12C12 and 1(11)A3H3 were selected.

Assay conditions for a sandwich-EIA to measure LPL and HTGL masses

The purified human PHP-LPL or -HTGL was dissolved in normal human serum (Flow Laboratories), pretreated for 30 min at 56°C, containing EDTA (1 mg/ml) and used as a standard LPL (0, 50, 100, 200, 300, and 400 ng/ml of serum) or HTGL (0, 100, 200, 400, 600, and 800 ng/ml of serum). The standard LPL (50 μl), HTGL (50 μl), preheparin plasma (50 μl), or PHP sample (50 μl) was placed in a test tube containing 50 μl of 0.5% Tween 20 and heparin (10 U/ml) solution in ice-cold water. First, 200 μl of β-galactosidase-labeled anti-LPL MAb [1(1)D2B2, 0.043 μg of IgG1/ml] was added to the above test tube and subsequently 200 μl of anti-LPL MAb linked with the cell walls [2(10)F8F9, 56.12 μg of IgG1/ml] was added, and the reaction mixture was incubated for 15 min at 37°C. Then, 2 ml of washing solution (2% NaCl and 0.1% Tween 20 solution) was added to the test tube and centrifuged at 2000 *g* for 10 min at 4°C. The supernatant was discarded and the pellet was resuspended with 2 ml of the washing solution. After centrifugation and discard of the supernatant, the pellet was resuspended in 500 μl of buffer A, and the β-galactosidase enzyme assay was started by addition of 100 μl of fluorescent substrate solution (0.3 mM 4-methyl-umbelliferyl-β-D-galactopyranoside in buffer A). The reaction mixture was incubated for 30 min at 37°C and the reaction was terminated by the addition of 1.5 ml of stop solution (0.1 M K_2HPO_4–NaOH buffer, pH 11.0). Fluorescence intensity was measured at an excitation of 365 nm and an emission of 450 nm using a Marcent Reader F-100 (Dainippon Pharmaceutical Co., Japan) with which the concentration of LPL mass is automatically calculated by reference to a standard calibration curve obtained from six points of LPL standards.

The immunoreactive mass of HTGL in PHP was quantified by the sandwich-EIA using β-galactosidase-labeled anti-HTGL MAb [2(4)F12C12, 0.041 μg of IgG1/ml] and anti-HTGL MAb linked with the cell walls [1(11)A3H3, 57.3 μg of IgG1/ml] using the same procedures as above. Concentration of HTGL mass was calculated by referring to a standard calibration curve obtained from six points of HTGL standards.

Electrophoresis and Western blotting

The samples were processed with 1.0% SDS in the presence of 33 mM dithiothreitol as described previously (8) and analyzed by a slab SDS-PAGE with 9% or 10% gel as described by Laemmli (17). Western blotting was carried out according to the method described in our previous paper (8). After SDS-PAGE analysis, proteins in the gel were electrophoretically transferred onto a nitrocellulose membrane at 200 mA for 12 h in a Tris-glycine buffer (0.02 M Tris, 0.15 M glycine, 20% methanol, 0.02% SDS, pH 8.3) (8, 18). The membrane was first blocked by incubation with 20 mM Tris-0.5 M NaCl buffer, pH 7.5 (TBS) containing 1% skim milk (DIFCO) for 1 h at 25°C. The membrane was reacted for 1 h at 37°C with 10 ml of a mouse anti-human PHP-LPL or anti-human PHP-HTGL MAb diluted $1:10^5$ in 20 mM Tris-0.5 M NaCl-0.05% Tween 20 buffer, pH 7.5 (TTBS) containing 1% skim milk. After washing with TTBS, the

membrane was incubated for 1 h at 37°C with 10 ml of biotinylated horse anti-mouse IgG antiserum in TTBS–1% skim milk, reacted for 1 h at 37°C with 10 ml of avidin-biotinylated horseradish peroxidase complexes (Vectastain ABC kit, PK-4002, Vector Laboratories, Burlingame, CA), and then stained with 4-chloro-1-naphthol according to the supplier's directions. A portion of the membrane was stained with 0.1% Amido Black in 10% acetic acid and 45% methanol, and destained in 2% acetic acid and 90% methanol (19).

Selective measurement of LPL and HTGL activities in PHP by SIIA

LPL and HTGL activities in PHP were measured by monitoring the residual lipolytic activity after the selective immunoinactivation of one of the two lipases with rabbit anti-human PHP-LPL and -HTGL antisera as described in our previous paper (8). This method was designated as selective immunoinactivation assay (SIIA). Three sets of the PHP sample–antiserum mixtures were prepared as follows. The PHP samples (25 μl each) were mixed with 5 μl of anti-human PHP-LPL antiserum, 5 μl of anti-human PHP-HTGL antiserum, or both antisera (5 μl each). The final volume of the mixtures was adjusted to 100 μl of 10 mM phosphate buffer (pH 7.0) containing 1 mM EDTA, 100 mM 6-aminocaproic acid, 3 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, and 10% glycerol. The mixtures were left to stand for at least 1 h on an ice bath. Without centrifuging, the residual lipase activity was measured with three different amounts (5, 10, and 20 μl) of the above enzyme mixtures under the best conditions for each LPL and HTGL assay. The reaction mixture contained 0.2 M Tris-HCl (pH 8.5), 5% BSA, 0.6 μmol of tri[9,10-^{3}H]olein (sp act 8.93 μCi/μmol) in the presence or absence of 1.7 μM pure apoC-II, 0.4% of gum arabic, 0.15 M NaCl, and the above enzyme solution; the total final volume was 0.30 ml. LPL activity was measured in the presence of apoC-II, but apoC-II was omitted for the measurement of HTGL activity. The reaction was started by the addition of the enzyme solution, and then the mixture was incubated for 30 min at 37°C. The released [9,10-^{3}H]oleate was determined by the liquid–liquid partitioning system (20). Units of enzyme activity were expressed as μmoles of free fatty acid released per h.

Other procedures

Triglyceride and cholesterol in plasma samples were measured by the enzymatic method using a Triglyceride E-Test kit (Wako Pure Chemical Co., Japan) and a Determiner TC-S kit (Kyowa Hakko, Japan) according to the supplier's directions. Apoproteins including A-I, A-II, B, C-II, and E were measured by radial immunodiffusion (RID) assay kits (Daiichi Pure Chemicals Co., Tokyo, Japan) according to the supplier's directions.

RESULTS

Properties of native MAbs directed against the purified human PHP-LPL and PHP-HTGL

To establish a sandwich-EIA for the quantitation of LPL mass, we obtained two types of MAbs that recognize different epitopes on the LPL enzyme. These are mouse anti-human PHP-LPL MAbs designated as 1(1)D2B2 and 2(10)F8F9. Similarly, we obtained two distinct mouse anti-human PHP-HTGL MAbs, 1(11)A3H3 and 2(4)F12C12, that recognize different epitopes on the HTGL enzyme. The titer and specificity of these four MAbs were determined by immunotitration experiments as shown in **Fig. 1**. Both anti-human PHP-LPL MAbs, 1(1)D2B2 and 2(10)F8F9, specifically immunoprecipitated LPL activity in the presence of rabbit anti-mouse IgG antibody as the second antibody, but they did not precipitate HTGL activity at all (Fig. 1A). The anti-human PHP-HTGL MAbs, 1(11)A3H3 and 2(4)F12C12, precipitated only HTGL activity, but not LPL activity (Fig. 1B). From the titration curves, it was estimated that the 1(1)D2B2 and

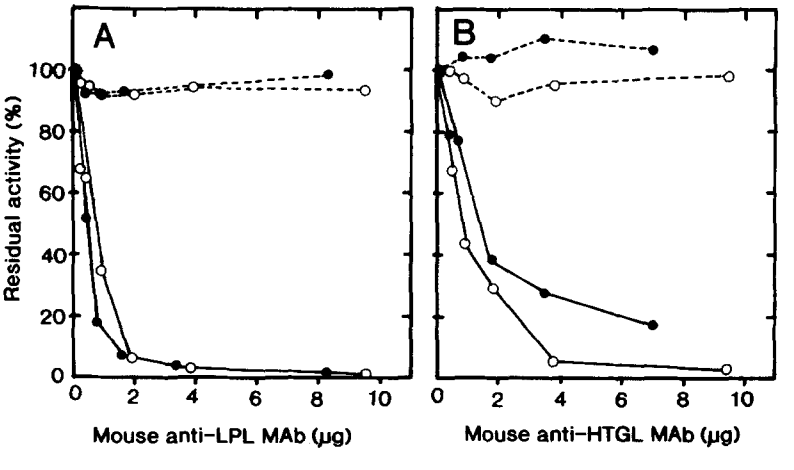

Fig. 1. Specific immunoprecipitation of LPL and HTGL catalytic activites with the corresponding native MAbs. Pure human PHP-LPL (227 ng of protein) and -HTGL (290 ng of protein) enzymes were incubated at 0°C for at least 1 h with the indicated amounts of anti-human PHP-LPL (A) and -HTGL MAbs (B) in the presence of rabbit anti-mouse IgG antiserum (80 μg of immunoglobulin) as the second antibody. After centrifugation, the enzyme activity in the supernatant was assayed. (A) Anti-human PHP-LPL MAbs, 1(1)D2B2 (●) and 2(10)F8F9 (○), were incubated with pure LPL enzyme (solid lines) and HTGL enzyme (dotted Lines). (B) Anti-human PHP-HTGL MAbs, 1(11)A3H3 (●) and 2(4) F12C12 (○), were incubated with pure HTGL enzyme (solid lines) and LPL enzyme (dotted lines).

2(10)F8F9 MAbs were each capable of precipitating 0.12 μg of pure LPL protein per μg of IgG1, and that the 1(11)A3H3 and 2(4)F12C12 MAbs were at least 0.04 μg and 0.07 μg of pure HTGL protein per μg of IgG1.

The specificity of native anti-human PHP-LPL and -HTGL MAbs was further ascertained by Western blot analysis, as shown in **Fig. 2** and **Fig. 3**. Three sets of the same sample preparations were first analyzed by slab SDS-PAGE with 9% gel and electrophoretically transferred to nitrocellulose membranes. When one of them was stained with Amido Black, it was confirmed that the proteins of all the samples were well transferred (Fig. 2A). Other membranes were used to determine the specificity of the 1(1)D2B2 (Fig. 2B) and 2(10)F8F9 MAbs (Fig. 2C). Both MAbs reacted with the LPL protein and showed a single band at 61 kD (lanes 1 and 2), but did not cross-react with other proteins in crude preparation (lane 2), antithrombin III (lane 3), pure HTGL protein (lane 4), or authentic standard proteins (lane 5). Similarly, three sets of the same preparations were used for the analysis of the specificity of anti-human PHP-HTGL MAbs. The proteins in all the samples were well transferred to a membrane (Fig. 3A). The 2(4)F12C12 (Fig. 3B) and 1(11)A3H3 MAbs (Fig. 3C) specifically reacted with HTGL protein and exhibited a single band at 65 kD (lanes 1 and 2), but did not cross-react with other proteins in crude preparation (lane 2), or authentic standard proteins (lane 3). Both anti-human PHP-HTGL MAbs did not react with LPL protein or antithrombin III at all (data not shown).

Properties of the modified MAbs

The anti-human PHP-LPL and -HTGL MAbs were chemically bound to bacterial cell walls as a carrier and to β-galactosidase to establish the sandwich-EIA methods. To determine whether or not the modified MAbs had the same properties as the corresponding native MAbs, immunotitration experiments were carried out using pure LPL and HTGL enzymes as antigens. The anti-human PHP-LPL MAbs linked to the cell walls, 1(1)D2B2 and 2(10)F8F9, specifically precipitated only LPL, but not HTGL activity as in the case where the corresponding native MAbs were used (**Fig. 4A**). Similarly, both anti-human PHP-HTGL MAbs linked with the cell walls specifically precipitated HTGL activity, but not LPL activity (Fig. 4B). In the case of β-galactosidase-labeled MAbs, their specificity was the same as that of the corresponding native MAbs (data not shown). The titer of anti-human PHP-LPL and -HTGL MAbs bound to the cell wall, however, was reduced to about one-fifth of the corresponding native MAbs (Fig. 4A and B).

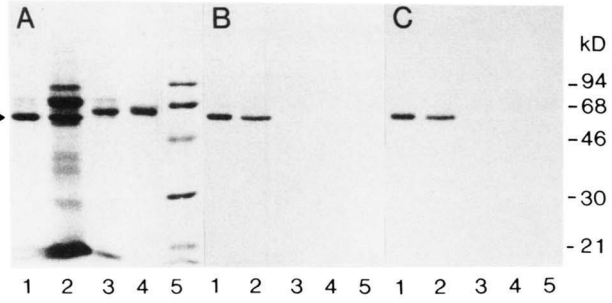

Fig. 2. Demonstration of the specificity of native anti-human PHP-LPL MAbs with Western blot analysis. The sample preparations were resolved by slab SDS-PAGE with 9% gel and electrophoretically transferred to a nitrocellulose membrane. (A): Protein bands stained with Amido Black. (B) and (C): Protein bands that specifically reacted with 1(1)D2B2 (B) and 2(10)F8F9 anti-LPL MAbs (C) were visualized by the procedure described in Materials and Methods. In each panel, lane 1, pure human PHP-LPL (2.5 μg); lane 2, the mixed crude preparation (25 μg) containing pure LPL (2.0 μg), human PHP (0.01 μl), and samples (10 μl) obtained through hydroxyapatite chromatography (8); lane 3, human antithrombin III (3.0 μg); lane 4, pure human PHP-HTGL (2.5 μg); and lane 5, authentic standard proteins (1.6–3.7 μg). The arrow shows the location of the LPL band.

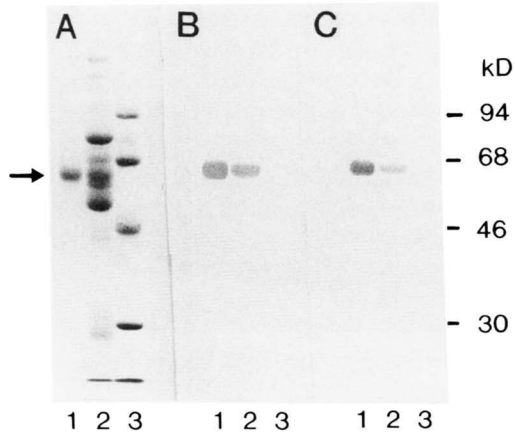

Fig. 3. Demonstration of the specificity of native anti-human PHP-HTGL MAbs with Western blot analysis. The sample preparations were resolved by slab SDS-PAGE with 9% gel and electrophoretically transferred to a nitrocellulose membrane. (A): Protein bands stained with Amido Black. (B) and (C): Protein bands that specifically reacted with 2(4)F12C12 (B) and 1(11)A3H3 anti-HTGL MAbs (C) were visualized by the procedure described in Materials and Methods. In each panel, lane 1, pure human PHP-HTGL (2.2 μg); lane 2, the mixed crude preparation containing pure HTGL (1.5 μg) and human PHP (16.7 μg) from which albumin was removed through Blue-Sepharose chromatography; lane 3, authentic standard proteins (1.6–3.7 μg). The arrow shows the location of the HTGL band.

Establishment of the sandwich-EIA and its specificity

The sandwich-EIA for LPL was performed using the combination of 1(1)D2B2 anti-LPL MAb linked with β-galactosidase and 2(10)F8F9 anti-LPL MAb linked with the cell wall. The sandwich-EIA for HTGL was carried out using the combination of 2(4)F12C12 anti-HTGL MAb linked with β-galactosidase and 1(11)A3H3 anti-HTGL MAb tagged with the cell wall. To determine the optimum incubation conditions for the sandwich-EIA, the time course of the immunoreactions was studied at 37°C with samples containing various concentrations of

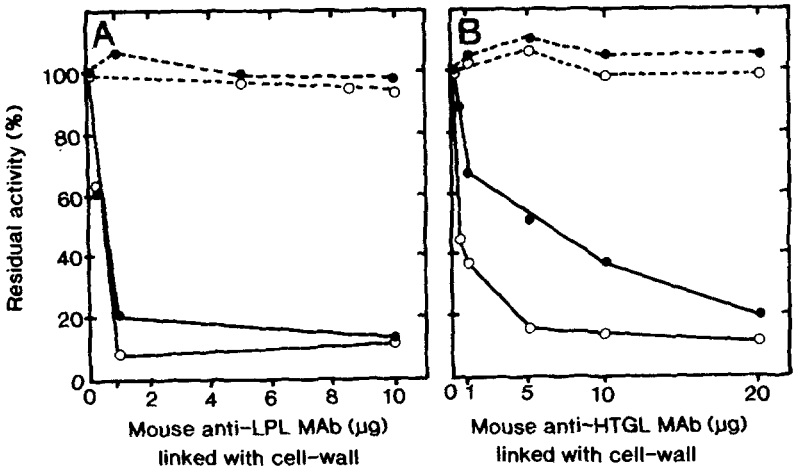

Fig. 4. Specific immunoprecipitation of LPL and HTGL catalytic activities with the corresponding MAbs linked with bacterial cell wall. Pure human PHP-LPL (34.5 ng of protein) and -HTGL (48.8 ng of protein) enzymes were reacted at 0°C for at least 1 h with the indicated amounts of anti-human PHP-LPL (A) and -HTGL MAbs (B) linked with bacterial cell wall. After centrifugation, the enzyme activity in the supernatant was assayed. (A) Anti-LPL MAbs linked with the cell wall, 1(1)D2B2 (●) and 2(10)F8F9 (○), were reacted with pure LPL enzyme (solid lines) and HTGL enzyme (dotted lines). (B) Anti-HTGL MAbs linked with cell wall, 1(11)A3H3 (●) and 2(4)F12C12 (○), were reacted with pure HTGL enzyme (solid lines) and LPL enzyme (dotted lines).

pure human PHP-LPL (0, 50, 100, 200, 300, and 400 ng/ml) and pure human PHP-HTGL (0, 100, 200, 400, 600, and 800 ng/ml) dissolved in control human serum. In immunoreactions for both LPL (**Fig. 5A**) and HTGL (Fig. 5B), reactions were linear until 15 min, then gradually diminished, and reached a plateau at 30–60 min. In both cases, when immunoreactive masses in the same samples were calculated by reference to the four standard curves obtained at each incubation time (15, 30, 60, 120 min), almost the same mass values were observed at each incubation time. Thus, the incubation time was chosen to be 15 min. In both reactions (Fig. 5A and B), the control human serum gave a very low fluorescence intensity (less than 150) in the absence of the added LPL or HTGL protein, indicating that the control human serum constituents did not interfere with present sandwich-EIA methods for LPL and HTGL. It should be noted that Tween 20 and heparin were added to final concentrations of 0.05% and 0.5 unit, respectively, in the assay to minimize nonspecific adsorption of LPL or HTGL to a glass test tube. These reagents did not interfere with the sandwich-EIA under the conditions used.

With the optimized assay conditions for the sandwich-EIA, standard calibration curves for LPL and HTGL were established. For standards, three different lots of the purified human PHP-LPL and -HTGL preparations were used. The standard curves for LPL (0–400 ng/ml) and HTGL (0–800 ng/ml) are shown in **Fig. 6A and B**. In both cases, standard curves from the three distinct samples did not show any significant differences. Fluorescence intensity was proportional to LPL or HTGL mass in the sample. Limit of detection was further determined in detail by analyzing mean ± 3SD of the fluorescence values measured with a low range of the standard LPL (0, 10, 20, 30, 40, and 50 ng/ml) and HTGL (0, 20, 40, 60, 80, and 100 ng/ml). The sample was repeatedly measured six times and the mean ± 3SD was calculated. In the absence of LPL or HTGL, fluorescence intensity (background) was 125.0 ± 25.8 (mean ± 3SD, n = 6) for LPL assay and 96.0 ± 20.4 (mean ± 3SD, n = 6) for HTGL assay, respectively. In the case of the LPL assay, fluorescence intensity (181.6 ± 26.7; mean ± 3SD, n = 6) obtained at 20 ng/ml did not overlap with the highest value of the background, and thus the limit of detection was estimated to be 20 ng/ml. For HTGL, under the same procedures, the limit of detection was estimated to be 60 ng/ml based on its fluorescence intensity (140.7 ± 10.1; mean ± 3SD, n = 6). These results indicate that the sensitivities for LPL and HTGL are 20–400 ng/ml and 60–800 ng/ml, respectively.

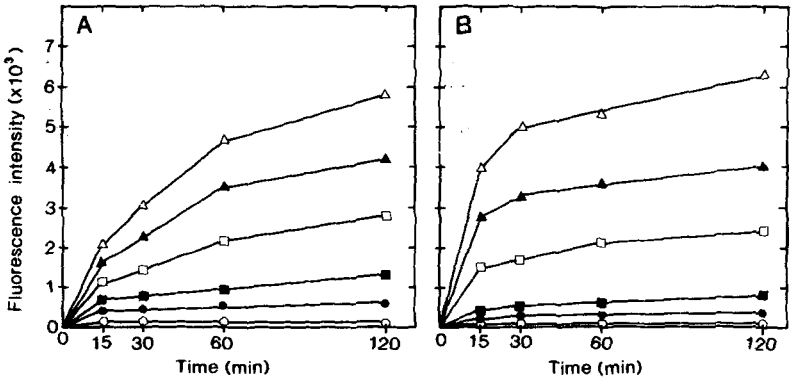

Fig. 5. Time course of immunoreaction of the sandwich-EIA for LPL (A) and HTGL (B). The sandwich-EIA methods were carried out as described in Materials and Methods. (A) An anti-LPL MAb (1(1)D2B2) linked with β-galactosidase and an anti-LPL MAb (2(10)F8F9) linked with the cell wall were reacted for the indicated time with pure LPL enzyme dissolved in normal human serum; (○), 0 ng/ml; (●), 50 ng/ml; (■), 100 ng/ml; (□), 200 ng/ml; (▲), 300 ng/ml; (△), 400 ng of pure LPL/ml. (B) An anti-HTGL MAb (2(4)F12C12) linked with β-galactosidase and an anti-HTGL MAb (1(11)A3H3) linked with cell wall were reacted for the indicated time with pure HTGL dissolved in normal human serum; (○), 0 ng/ml; (●), 100 ng/ml; (■), 200 ng/ml; (□), 400 ng/ml; (▲), 600 ng/ml; (△), 800 ng of pure HTGL/ml.

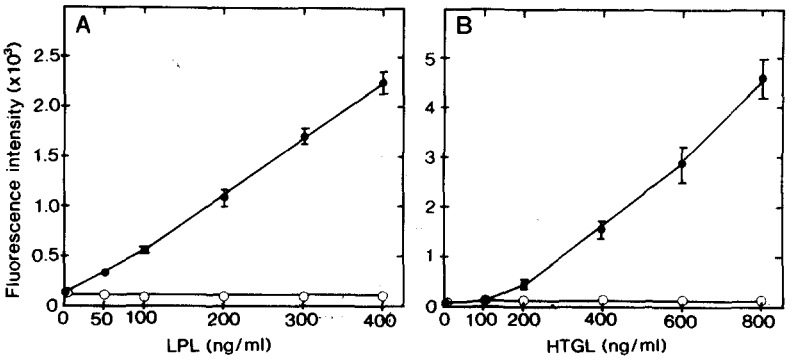

Fig. 6. Standard curves of LPL (A) and HTGL (B) masses. (A) Three distinct purified human PHP-LPL preparations, with specific activities of 42.1, 46.5, and 45.8 mmol/h per mg, were dissolved in control human serum to the concentrations indicated on the abscissa. The immunoreactive mass of LPL was measured by the sandwich-EIA using the combination of β-galactosidase-labeled 1(1)D2B2 and 2(10)F8F9 linked with the cell wall (●) or using the combination of the same MAbs such as β-galactosidase-labeled 1(1)D2B2 and 1(1)D2B2 linked with the cell wall (○). (B) Three distinct purified human PHP-HTGL preparations, with specific activities of 18.7, 18.6, and 17.0 mmol/h per mg, were dissolved in control human serum to the indicated concentrations. The immunoreactive mass of HTGL was measured by the sandwich-EIA using the combination of β-galactosidase-labeled 2(4)F12C12 and 1(11)A3H3 linked with the cell wall (●) or using the combination of the same MAbs such as β-galactosidase-labeled 2(4)F12C12 and 2(4)F12C12 linked with the cell wall (○). Each point represents the mean ± SD of three preparations.

When the combination of the same MAbs such as β-galactosidase-labeled 1(1)D2B2 anti-LPL MAb and 1(1)D2B2 MAb linked with the cell wall was used to detect LPL mass, there was no increase in fluorescence intensity, indicating that they recognize the same epitope on the LPL molecule and could not sandwich the LPL protein at all (Fig. 6A). Similarly, the combination of the same anti-HTGL MAbs could not sandwich the HTGL protein at all (Fig. 6B).

Stability of standard LPL and HTGL, and PHP samples

Pure human PHP-LPL and -HTGL were dissolved in human serum, and they were used as the standard LPL and HTGL preparations. The effect of temperature on the stability of the standard LPL and HTGL samples was examined and the results are shown in **Fig. 7**. The standard LPL (200 ng/ml) and HTGL (400 ng/ml) samples were preincubated for the indicated time at 0°C, 4°C, 25°C, and 37°C. The LPL (Fig. 7A) and HTGL (Fig. 7B) samples were quite stable for at least 60 min at 0°C, 4°C, and 25°C. However, the preincubation at 37°C for 30 min decreased the immunoreactivity of both lipases by 15–20%. When the same standard LPL and HTGL samples were stored at −80°C, they were stable for at least 1 year. The results on the stability of human PHP samples containing LPL or HTGL were essentially the same as those of the standard preparations.

The effect of freezing and thawing on human PHP samples was investigated. Human PHP samples were freshly obtained from three individuals. An aliquot of each sample was kept at 0°C and the remaining preparations of the same samples were frozen at −80°C. Freezing and thawing cycles were repeated one to five times, and the analysis was started within 1 h. LPL mass in the three fresh samples was 341.4, 302.4, and 205.8 ng/ml, and after at least five freezing–thawing cycles, the LPL mass value remained constant, 98–109%, 93–103%, and 96–106%, respectively. HTGL mass in the same samples was 1356.8, 1677.8, and 1105.0 ng/ml, and after at least

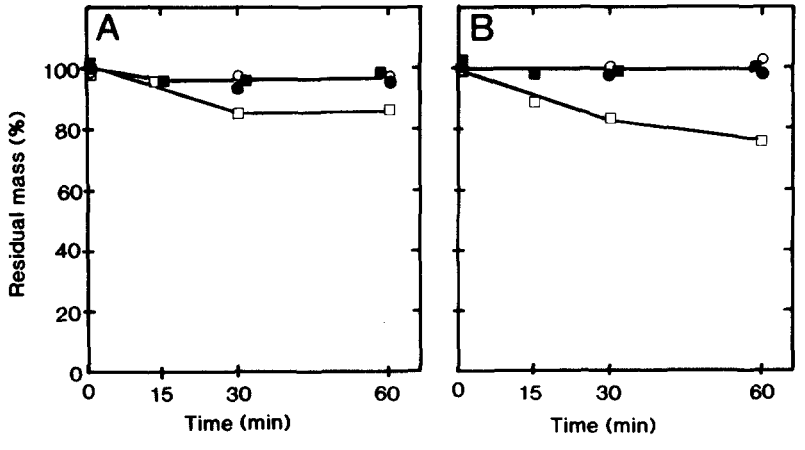

Fig. 7. Effect of temperature on the stability of standard LPL (A) and HTGL (B). In (A) and (B), pure human PHP-LPL (200 ng/ml) and -HTGL (400 ng/ml), dissolved in control human serum, were preincubated for the indicated time at 0°C (●), 4°C (○), 25°C (■), and 37°C (□). After preincubation, the remaining LPL and HTGL masses were assayed by the sandwich-EIA. Each point represents the average of duplicates, and the duplicate values differed by less than 5%.

TABLE 1. Precision test for the determination of LPL and HTGL masses by the sandwich-EIA

	LPL				HTGL			
	Sample[a]	Assay	Mean ± (SD)	Coefficient Variation	Sample[b]	Assay	Mean ± (SD)	Coefficient Variation
		n	*ng/ml*	%		*n*	*ng/ml*	%
Intra-assay	A	10	75.5 (2.26)	2.99	D	10	154.5 (8.38)	5.4
	B	10	150.8 (4.19)	2.78	E	10	314.6 (14.85)	4.7
	C	10	293.5 (7.08)	2.41	F	10	630.5 (22.62)	3.6
Inter-assay	A	5	76.6 (2.06)	2.65	D	5	151.5 (5.42)	3.6
	B	5	147.5 (5.79)	3.95	E	5	304.2 (20.01)	6.6
	C	5	296.4 (11.90)	4.61	F	5	606.2 (35.52)	5.5

[a]Pure human PHP-LPL was mixed with control human serum to give final concentrations of 75 (A), 150 (B), and 300 ng/ml (C).
[b]Pure human PHP-HTGL was mixed with control human serum to give final concentrations of 150 (D), 300 (E), and 600 ng/ml (F).

five times freezing-thawing, HTGL mass also remained constant, 99-105%, 98-101%, and 87-104% for three groups of samples. These results indicate that at least five freezing-thawing cycles did not affect LPL and HTGL immunoreactive mass levels in human PHP.

Validation of the sandwich-EIA for LPL and HTGL

The validity of the sandwich-EIA for LPL and HTGL quantification was examined by precision, recovery, and dilution tests. The precision studies on LPL and HTGL were carried out using three distinct samples containing high, medium, and low concentrations of the corresponding enzyme. As shown in **Table 1**, the intra- and inter-assay coefficients of variation for the three LPL samples were less than 3% and 5%, respectively, and those for the HTGL samples were less than 6% and 7%, respectively. Recovery tests were performed using five distinct samples made by further addition of the purified human PHP-LPL or -HTGL to the original human PHP samples. The average recoveries of the five LPL and HTGL samples were 98.8 ± 5.75% and 97.5 ± 1.70% (mean ± SD), respectively. Dilution tests were carried out using three distinct PHP samples containing high, medium, and low concentrations of LPL or HTGL. In all LPL samples (**Fig. 8A**), linear dose-response curves were obtained with the range of 0-300 ng/ml, and linear curves for all HTGL samples were obtained with the range of 0-700 ng/ml (Fig. 8B).

The validity of the sandwich-EIA for LPL and HTGL was further examined in the presence of a high concentration of triglyceride and other substances including hemoglobin, bilirubin, blood urea nitrogen, uric acid, and creatinine. The results are shown in **Table 2**. The quantifications of LPL and HTGL masses were not significantly affected by either hypertriglyceridemic plasma (up to 2900 mg of triglyceride/dl) or high concentrations of triglyceride (up to 5000 mg/dl) from Intralipos. No other substances disturbed the sandwich-EIA for the quantification of LPL and HTGL.

Application of sandwich-EIA to clinical samples

We investigated the relationship between heparin dose and the released enzyme levels in PHP in five normolipi-

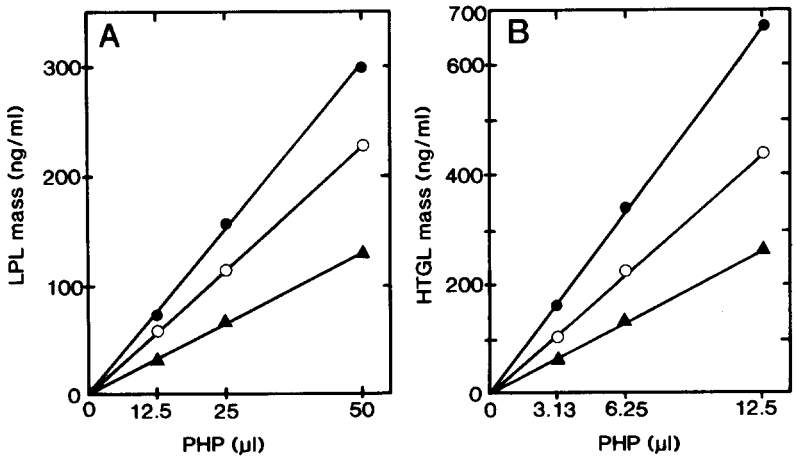

Fig. 8. Dose-response lines of LPL and HTGL masses measured by the sandwich-EIA. (A) Three PHP samples containing LPL concentration of 295 ng/ml (●), 231 ng/ml (○), and 133 ng/ml (▲) were serially diluted with control human serum. (B) Three PHP samples containing HTGL concentration of 672 ng/ml (●), 440 ng/ml (○), and 267 ng/ml (▲) were serially diluted with control human serum. The immunoreactive masses of LPL and HTGL in PHP samples were measured by the sandwich-EIA. Each point represents the average of duplicates, and the duplicate values differed by less than 5%.

TABLE 2. Validation of the sandwich-EIA with various substances

Substances	Concentration	LPL	HTGL
	mg/dl	*% of control*	
Hypertriglyceridemic plasma	2900[a]	101.2	93.2
Intralipos	5000[a]	94.8	105.8
Hemoglobin	1000	106.4	111.2
Bilirubin	20	99.1	99.5
Blood urea nitrogen	200	105.1	101.2
Uric acid	20	99.2	101.4
Creatinine	20	103.9	95.5

PHP samples containing LPL (200 ng/ml) and HTGL (400 ng/ml) were analyzed in the absence and presence of the indicated substances by the sandwich-EIA for LPL and HTGL. Percentage of control was calculated by taking the mass values measured in the absence of these substances as 100%. Measurements represent the average of duplicates.

[a]These values are expressed as triglyceride concentration.

demic subjects. PHP samples were obtained 10 min after the injection of heparin at doses of 10, 30, and 60 IU/kg to each subject. Immunoreactive masses as well as activities of LPL and HTGL in PHP were determined by the sandwich-EIA and SIIA. The results obtained by the sandwich-EIA are shown in **Fig. 9**. For the varying heparin doses, the maximum release of the two lipases (both mass and activity) was obtained at a heparin dose of 60 IU/kg. Compared to the maximal mass values at 60 IU/kg as 100%, a heparin dose of 30 IU/kg gave a near-maximum response (88.9 ± 11.0%, mean ± SD) for LPL and 85.6 ± 8.6% for HTGL, while a dose of 10 IU/kg was 51.0 ± 5.0% for LPL and 58.7 ± 8.6% for HTGL.

LPL and HTGL masses in PHP, obtained at 30 IU/kg of heparin, from 34 normolipidemic (normal) and 20 hypertriglyceridemic subjects were measured by the sandwich-EIA. LPL and HTGL activities in PHP from the same subjects were determined by SIIA (8). To compare both methods, the sandwich-EIA and SIIA, enzyme activities measured by SIIA were also expressed as mass values calculated by dividing the enzyme activities by the specific activity of the purified human PHP-LPL (45 mmol/h per mg) and PHP-HTGL (18 mmol/h per mg). This way is effective to simply judge whether or not the mass values measured by the sandwich-EIA are equivalent to those calculated from the native lipase activities by checking a slope value (a typical value = 1.0) from linear regression analysis. A comparison of LPL mass, obtained by the two methods above, in PHP from normolipidemic subjects yielded a high correlation coefficient of 0.945 ($P<0.001$) and a slope of 0.973 (**Fig. 10A**). In the case of HTGL, there was also a high correlation ($r = 0.932$, $P<0.001$), with a slope of 0.902 (**Fig. 11A**), between the values obtained by the two methods above. These results indicate that the LPL and HTGL mass values determined by our sandwich-EIA are almost equivalent to the mass values of both native enzymes and can also represent their enzyme activities. For the whole group of 34 normolipidemic subjects, the mean (± SD) values of mass and activity in PHP were 223 ± 66 ng/ml and 10.1 ± 2.9 μmol/h per ml, respectively, for LPL, and were 1456 ± 469 ng/ml and 26.4 ± 8.7 μmol/h per ml, respectively, for HTGL. In 20 hypertriglyceridemic subjects, a comparison of LPL mass and activity gave a high correlation ($r = 0.989$, $P<0.001$) and a slope of 0.936 (Fig. 10B); a comparison of HTGL mass and activity yielded a high correlation ($r = 0.954$, $P<0.001$) and a slope of 0.957 (Fig. 11B). Among 20 hypertriglyceridemic subjects, two individuals exhibited zero activity of LPL in PHP and their LPL mass values were also not detected by the sandwich-EIA (Fig. 10B). For the whole group of 20 hypertriglyceridemic subjects, the mean (± SD) values of mass and activity were 129 ± 125 ng/ml and 5.7 ± 5.7 μmol/h per ml for LPL, respectively; 1124 ± 702 ng/ml and 21.1 ± 12.6 μmol/h per ml for HTGL, respectively.

We also measured the immunoreactive mass and activity of LPL and HTGL in the preheparin plasma obtained from the five normolipidemic subjects. The immunoreactive mass and activity of LPL were 33.36 ± 4.69 ng/ml and 0.054 ± 0.018 μmol/h per ml, respectively, and those of HTGL were 24.05 ± 12.03 ng/ml and 0.054 ± 0.16 μmol/h per ml, respectively. In a previous paper (6), it was reported that human preheparin plasma contained little total lipase activity (less than 0.6 μmol/h per ml) including both LPL and HTGL.

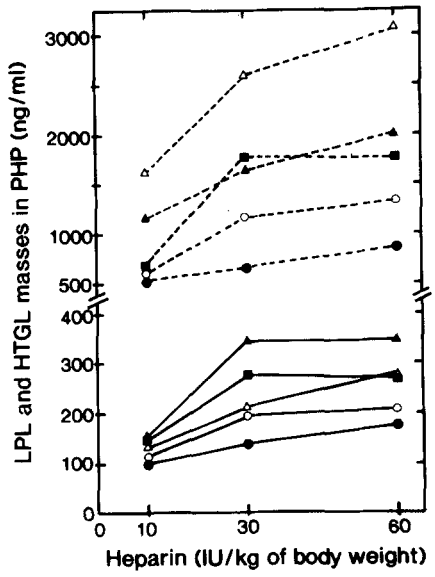

Fig. 9. Dose-response curves of LPL and HTGL masses in PHP for the varying heparin doses. PHP samples were obtained from five normolipidemic subjects 10 min after injection of heparin at doses of 10, 30, and 60 IU/kg at an interval of at least 3 days. Heparin administration to each subject was done in a random sequence. LPL and HTGL masses were measured by the sandwich-EIA. Five individuals are expressed as symbols of ●, ○, △, ▲, and ■. Solid lines represent LPL and dotted lines are HTGL.

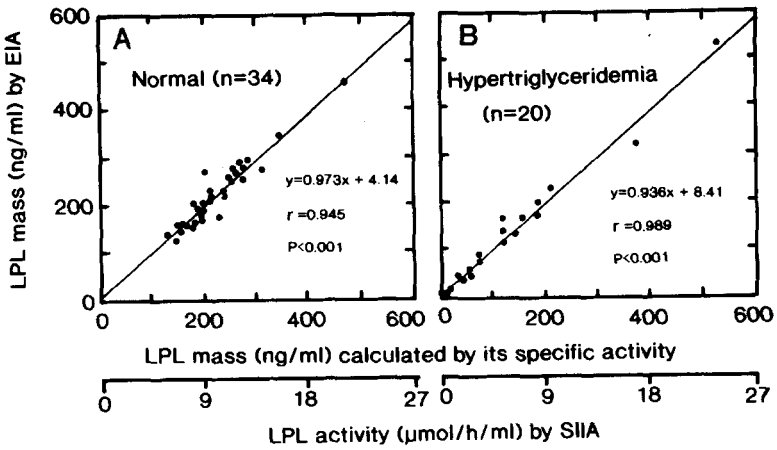

Fig. 10. Correlation between LPL mass and activity in PHP from normolipidemic (A) and hypertriglyceridemic subjects (B). PHP samples were obtained 10 min after injection of 30 IU/kg of heparin. In (A) and (B), LPL mass was determined by the sandwich-EIA. LPL activity was measured by SIIA, and LPL mass was calculated by using the specific activity (45 mmol/h per mg) or pure human PHP-LPL enzyme.

DISCUSSION

In the present study, we demonstrate the successful development of a sandwich-EIA for the determination of immunoreactive masses of LPL and HTGL in human PHP samples using MAbs directed against the corresponding enzymes. To determine the immunoreactive masses of LPL and HTGL in human PHP, it is best to use antibodies directed against both enzymes purified from human PHP as the same starting material. We have succeeded in obtaining anti-human PHP-LPL and -HTGL MAbs raised in mice against the corresponding enzymes purified from human PHP. Moreover, we could obtain two types of anti-LPL MAbs that recognize different epitopes on the LPL molecule and also two distinct types of anti-human PHP-HTGL MAbs as in LPL. The monospecificity of the obtained MAbs to the corresponding enzymes was ascertained by both immunoprecipitation of the native enzyme and Western blotting techniques. The anti-human PHP-LPL MAbs specifically reacted with LPL, but not with HTGL or antithrombin III. Also, the anti-human PHP-HTGL MAbs were specific to HTGL only.

The present sandwich-EIA method for LPL has been established for the first time by using the combination of two distinct types of anti-human PHP-LPL MAbs and the purified human PHP-LPL as standard protein. Similarly, the sandwich-EIA for HTGL was developed. One of the MAbs was labeled with β-galactosidase and the other was linked with the bacterial cell wall, and those were used as an enzyme-linked MAb and an insolubilized MAb. One of the characteristics of our sandwich-EIA is the use of MAb linked with the bacterial cell wall, which retained a high immunoreactive capacity to precipitate the native enzyme by low-speed centrifugation instead of antibody immobilized on the surface of a plastic plate in the conventional ELISA. In this way, it was possible to rapidly quantify immunoreactive masses of LPL and HTGL in human plasma with a high degree of sensitivity by directly mixing plasma samples with a β-galactosidase-labeled MAb and MAb linked with the cell wall in a tube.

The validity of the present sandwich-EIA for LPL and

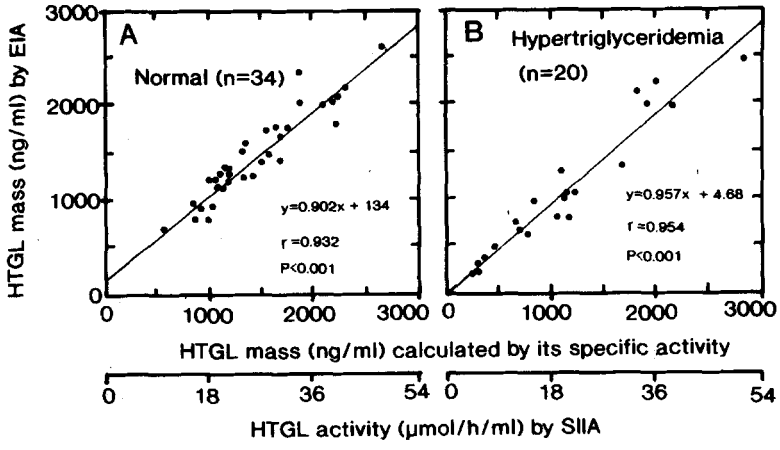

Fig. 11. Correlation between HTGL mass and activity in PHP from normolipidemic (A) and hypertriglyceridemic subjects (B). PHP samples were obtained 10 min after injection of 30 IU/kg of heparin. In (A) and (B), HTGL mass was determined by the sandwich-EIA. HTGL activity was measured by SIIA, and HTGL mass was calculated by using the specific activity (18 mmol/ h per mg) of pure human PHP-HTGL enzyme.

HTGL was verified by the experimental observations from precision (inter- and intra-assay), recovery, and dilution tests. Using the procedures described in this study, we could uniformly obtain inter- and intra-assay coefficients of variation of less than 6% for determinations of LPL and HTGL immunoreactive masses in human PHP. When LPL and HTGL enzymes were examined, either in purified forms or present in human PHP samples, all had linear dose-response curves. Moreover, the present sandwich-EIA is not affected by high triglyceride, urea nitrogen, uric acid, or creatinine levels in human PHP, indicating that this method is applicable to the determination of LPL and HTGL masses in PHP samples from subjects with hypertriglyceridemias or renal failure as well as normolipidemic subjects.

In general, the analysis of LPL and HTGL in human PHP appears to be important for understanding the pathogenesis of primary or secondary hypertriglyceridemias. Sampling of PHP is usually done 10–20 min after injection of a heparin dose of 10–100 IU/kg. Maximal response is obtained at a heparin dose of 100 IU/kg (6, 21) or 75 IU/kg (22) for the release of both lipase activities. In our study, a heparin dose of 60 IU/kg gave maximal release of both lipases among three doses of heparin (10, 30, and 60 IU/kg), whereas 30 IU/kg of herapin was as effective as 60 IU/kg for evaluating both mass and activity levels in PHP by the sandwich-EIA and SIIA. In fact, it was possible to clearly discriminate normal and heterozygote for LPL deficiency in a family pedigree with a homozygote for LPL deficiency by quantifying LPL mass and activity in PHP obtained at the same dose (Y. Ikeda, et al., unpublished results) (23). In the present study, PHP samples obtained at 30 IU/kg of heparin from normolipidemic and hypertriglyceridemic subjects were analyzed by both sandwich-EIA and SIIA. In normolipidemic subjects, both lipase mass levels measured by the sandwich-EIA were found to correlate well with the corresponding enzyme catalytic activities measured by SIIA (8), supporting the present sandwich-EIA as being valid. Our data indicate that the specific activity of normal LPL and HTGL in PHP is estimated to be 45 mmol/h per mg and 18 mmol/h per mg, respectively, under the conditions used in good agreement with the specific activity of both native lipases purified from human PHP. In the patients with hypertriglyceridemia, we could not find a significant difference between immunoreactive mass and catalytic activity in either LPL or HTGL.

It is suggested that LPL in PHP is derived from a number of different tissues including adipose tissue, heart, and skeletal muscle (3, 4, 24). So far, it is unclear whether or not LPL released into PHP has a tissue specificity in its cross-immunoreactivity, although LPL purified from human PHP yields a single band at 61 kD on SDS-PAGE (8). Therefore, it seems to be of importance to use MAbs that are capable of immunoprecipitating almost all of the native LPL enzyme in human PHP for an EIA to quantify LPL. In addition, there is a distinct species difference about the molecular nature of LPL: human LPL from PHP is catalytically active in a monomeric form (8), whereas rat LPL (25) and bovine milk LPL (26) are functionally active as dimers. Thus, the best way to quantify human LPL might be by using a homogeneous system utilizing polyclonal antibody or MAbs directed to human PHP-LPL as a standard. In this study, we have succeeded in establishing a sandwich-EIA for LPL using the anti-human PHP-LPL MAbs that were capable of precipitating more than 90% of the native LPL in human PHP and the pure human PHP-LPL as a standard. Others have reported an ELISA of LPL in human PHP using a single MAb directed against human milk LPL (13) or bovine milk LPL (14). An ELISA with an anti-human milk-LPL MAb failed to detect the immunoreactive mass of LPL in human PHP samples, but it could detect only LPL partially purified by heparin-agarose chromatography (13). Interestingly, LPL mass in human PHP samples was measured by an ELISA using a single anti-bovine milk-LPL MAb (5D2) and purified bovine milk LPL as a standard (14). In that study, however, the experimental data on the cross-reactivity of the 5D2 MAb to human PHP-LPL and the validity of ELISA were not described in detail as they are here. That ELISA method was based on sandwiching the LPL enzyme with the 5D2 MAb bound to a microtiter well and the same 5D2 MAb linked with horseradish peroxidase, indicating that the ELISA is applicable for a dimerized form of LPL with two intact epitopes for the 5D2 MAb. In our data, however, the combinations of the same anti-human PHP-LPL MAbs could not sandwich either purified LPL or LPL in human PHP at all, because the same MAbs competed for the same epitope on the LPL molecule. The LPL mass level (196 ± 59 ng/ml), reported by Babirak et al. (14), in PHP obtained at 60 IU/kg of heparin from normal subjects (n = 34), is similar to that (223 ± 66 ng/ml; n = 34) obtained at 30 IU/kg of heparin by us. However, it should be noted that the cross-reactivity of the antibody raised against bovine milk LPL to human PHP-LPL is a matter of controversy and that a polyclonal antibody (27, 28) or MAb to bovine milk LPL (29) exhibited only weak cross-reactivity to human PHP-LPL. Our MAbs directed against human PHP-LPL only partially reacted with the purified bovine milk LPL (Ikeda, Y., Z. Tsutsumi, A. Takagi, and A. Yamamoto, unpublished observation). In contrast, a solid-phase ELISA using a polyclonal antibody to bovine milk LPL has successfully been applied to measure LPL mass from rat tissue extracts (30). Recently, the latter ELISA system was used to determine LPL mass in human pre-heparin plasma and PHP (obtained at 60 IU/kg of

heparin) by using bovine milk LPL as a standard, and LPL mass values in PHP from eight normal subjects were reported to be 75–145 ng/ml, which is almost half that of our data; those of preheparin plasma were 0–25 ng/ml (31). In that study, Kern et al. indicated a possibility that the anti-bovine milk LPL polyclonal antibodies reacted in a different manner between bovine milk LPL and human LPL, and thus the true mass of LPL in human plasma may be different.

Our sandwich-EIA methods for LPL and HTGL are sufficiently rapid, accurate, and specific for the determination of the corresponding enzyme masses in either preheparin plasma or PHP samples from hypertriglyceridemic as well as normolipidemic human subjects. This study demonstrates that the molecular nature of both lipases in human PHP can be studied by measuring both mass and activity with our sandwich-EIA and SIIA.

We thank Drs. Shinzo Nishimura, Hiroo Ishii, and Noriyuki Sunahara for their excellent technical assistance. This research was supported in part by grants No. 01580206 and No. 61440053 from the Ministry of Education, Science and Culture of Japan.

Manuscript received 5 March 1990 and in revised form 25 June 1990.

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