Analytical performance of a sandwich enzyme immunoassay for preβ1-HDL in stabilized plasma

Takashi Miida,[1],* Osamu Miyazaki,[†] Yasushi Nakamura,[†] Satoshi Hirayama,[§] Osamu Hanyu,[§] Isamu Fukamachi,[†] and Masahiko Okada*

Division of Clinical Preventive Medicine,* Department of Community Preventive Medicine, Niigata University Graduate School of Medical and Dental Sciences, Asahimachi 1-757, Niigata, Niigata 951-8510, Japan; Daiichi Pure Chemicals,[†] Diagnostics Research Laboratories, Tokai Research Group, Muramatsu 2117, Tokai, Ibaraki 319-1182, Japan; and Division of Endocrinology and Metabolism,[§] Department of Homeostatic Regulation and Developments, Niigata University Graduate School of Medical and Dental Sciences, Asahimachi 1-757, Niigata, Niigata 951-8510, Japan

Abstract We have established an immunoassay for preβ1-HDL (the initial acceptor of cellular cholesterol) using a monoclonal antibody, MAb55201. Because preβ1-HDL is unstable during storage, fresh plasma must be used for preβ1-HDL measurements. In this study, we describe a method of stabilizing preβ1-HDL, and evaluate the analytical performance of the immunoassay for preβ1-HDL. Fresh plasma was stored under various conditions with or without a pretreatment consisting of a 21-fold dilution into 50% (v/v) sucrose. Preβ1-HDL concentration was measured by immunoassay. In nonpretreated samples, preβ1-HDL decreased significantly from the baseline after 6 h at room temperature. Although preβ1-HDL was more stable at 0°C than at room temperature, it increased from 30.2 ± 8.5 (SE) to 56.5 ± 5.5 mg/l apolipoprotein A-I (apoA-I) ($P < 0.001$) in hyperlipidemics, and from 18.4 ± 1.2 to 37.9 ± 3.3 mg/l apoA-I ($P < 0.001$) in normolipidemics after 5-day storage. After 30-day storage at −80°C, preβ1-HDL increased from 29.0 ± 4.0 to 38.0 ± 5.7 mg/l apoA-I ($P < 0.001$) in hyperlipidemics, whereas it did not change in normolipidemics. In pretreated samples, preβ1-HDL concentration did not change significantly under any of the above conditions. Moreover, preβ1-HDL concentrations determined by immunoassay correlated with those determined by native two-dimensional gel electrophoresis (n = 24, $r = 0.833$, $P < 0.05$).⬛ An immunoassay using MAb55201 with pretreated plasma is useful for clinical measurement of preβ1-HDL.—Miida, T., O. Miyazaki, Y. Nakamura, S. Hirayama, O. Hanyu, I. Fukamachi, M. Okada. **Analytical performance of a sandwich enzyme immunoassay for preβ1-HDL in stabilized plasma.** *J. Lipid Res.* 2003. 44: 645–650.

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A large number of studies have demonstrated that a high level of HDL is a negative risk factor for coronary artery disease (1–5). These observations are consistent with the hypothesis that HDL removes excess cholesterol from peripheral tissues (6). Clinically, HDL-cholesterol and apolipoprotein A-I (apoA-I) are used as markers for the quantity of HDL particles in plasma (7–9). However, the various HDL subfractions differ in their ability to remove cellular cholesterol (10–12). Therefore, researchers have sought a method to specifically measure these subfractions (13–17). One of these subfractions, preβ1-HDL, is more anti-atherogenic than other subfractions.

Preβ1-HDL contains apoA-I, phospholipid, and a small amount of free cholesterol (11). In cell culture systems, preβ1-HDL captures free cholesterol from cell membranes within a few minutes (11, 18–19). This property of preβ1-HDL is similar to that of reconstituted lipid-poor HDL (20), an experimental model of nascent HDL. Although preβ1-HDL comprises only a small proportion of the total apoA-I in healthy subjects (14, 21–23), its level changes significantly in coronary artery disease (14, 24), obesity (23), and hyperlipidemia (21, 25–27), as well as in patients treated with lipid-lowering therapies (21, 27). Preβ1-HDL concentrations are currently measured in a limited number of clinical laboratories by native two-dimensional (2D)-gel electrophoresis (14, 23–27). This method is not well suited for widespread use in clinical laboratories because it is expensive, time-consuming, and requires a significant amount of technical skill. Another obstacle encountered in the clinical laboratory is that preβ1-HDL is unstable during storage (14, 28), so that measurements should be made only on fresh plasma samples. We have recently established a sandwich enzyme immunoassay using a monoclonal antibody (MAb55201)

Abbreviations: 2D-gel, two-dimensional gel; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

[1] To whom correspondence should be addressed.
e-mail: miida@med.niigata-u.ac.jp

that is specific for preβ1-HDL (15). At present, this immunoassay is open to researchers. A Japanese commercial laboratory (SRL, Tachikawa, Japan) has been measuring preβ1-HDL concentrations in clinical or experimental samples using this assay. In this study, we describe a method for stabilization of preβ1-HDL by pretreatment, and we evaluate the analytical performance of the immunoassay for preβ1-HDL measurement.

MATERIALS AND METHODS

Study protocol

Blood samples were obtained from 20 volunteers (12 men and 8 women, aged 26 to 68 years), or 24 outpatients (13 men and 11 women, aged 22 to 76) at our institutions. The former group consisted of 10 normolipidemics and 10 hyperlipidemics (four hypercholesterolemics, three hypertriglyceridemics, and three combined hyperlipidemics). The latter group consisted of 11 normolipidemics, and 13 hyperlipidemics (six hypercholesterolemics, three hypertriglyceridemics, and four combined hyperlipidemics). All subjects willingly gave informed consent before entering into this study. The study protocol was approved by the ethical committee of our institution. Serum cholesterol concentrations were 3.83–7.03 mmol/l in the volunteers and 3.34–7.27 mmol/l in the outpatients; triglyceride concentrations were 0.45–4.10 mmol/l in the volunteers and 0.55–5.34 mmol/l in the outpatients.

Venous blood was drawn from subjects who had fasted for at least 12 h. The blood was immediately mixed with an anticoagulant (EDTA-K_2, 1 g/l) in a glass tube, and chilled on ice water. Plasma was separated at 0°C, placed into screw-capped tubes, and stored under different conditions before assaying for preβ1-HDL. In some experiments, LCAT activity was inhibited by the addition of 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) to a final concentration of 2 mmol/l. In some experiments, whole blood was stored under the indicated condition, and plasma was separated later. Some fresh plasma samples were pretreated by 1:20 (v/v) dilution with 50% sucrose solution (stabilizing buffer), mixed thoroughly, and stored under the same conditions as the nonpretreated samples.

The preβ1-HDL levels of all subjects were measured by the sandwich enzyme immunoassay technique described below (15). For some outpatients, preβ1-HDL levels were also determined by native 2D-gel electrophoresis. The preβ1-HDL concentrations obtained by these two methods were then compared.

Sandwich enzyme immunoassay

First, a 96-well plastic plate was coated with a monoclonal antibody (MAb 55201) specific for preβ1-HDL (Daiichi Pure Chemicals, Tokyo, Japan, miyazaki-o@daiichichem.co.jp) (15). Then, plasma was diluted 2,121-fold with 1% BSA-PBS. Diluted plasma (sample) or purified human apoA-I (standard) was then added to each well and incubated at room temperature for 1 h. The wells were washed three times with 0.1% BSA-PBS, and the adsorbed preβ1-HDL was incubated with a horseradish peroxidase-coupled secondary antibody (goat anti-human apoA-I polyclonal antibody). The wells were washed again with 0.1% BSA-PBS, and o-phenylenediamine and H_2O_2 in citrate buffer were added to the wells. The amount of preβ1-HDL in the wells was determined by measurement of the absorbance at 492 nm. The absorbance curves in the diluted samples were parallel to those obtained with the purified apoA-I. The coefficient of variation was 3.1% to 5.3% for individual analytical runs, and 4.9% to 9.1% between different analytical runs.

In the preliminary experiments, we confirmed that this immunoassay measures most apoA-I of the small HDL fraction as preβ1-HDL (15). Plasma lipoproteins were separated by gel chromatography with a FPLC system (Amersham Pharmacia Biotech). Then, we determined apoA-I and preβ1-HDL concentrations in each fraction by two immunoassays. Either polyclonal anti-apoA-I antibody or MAb55201 was used as a capture antibody. The high preβ1-HDL peak was detected in the small apoA-I-containing lipoprotein with molecular mass less than 67 kDa (15). In this fraction, preβ1-HDL/apoA-I ratio was 80–90%.

Native 2D-gel electrophoresis

Fresh plasma was separated in the first dimension by electrophoresis on 0.75% agarose gels, and then in the second dimension by electrophoresis on gradient (2% to 15%) polyacrylamide gels (14, 21, 25–28). The separated HDL subfractions in the polyacrylamide gels were then electroblotted onto nitrocellulose membranes. The membranes were probed with a ^{125}I-labeled goat anti-human apoA-I polyclonal antibody and autoradiographed. Using the autoradiogram as a guide, we clipped the HDL subfractions out of the membranes and determined the amount of radioactivity in the subfractions by γ spectrometry. The relative concentration of each subfraction was expressed as a percentage of the total apoA-I concentration. The absolute concentration was calculated by multiplying the relative concentration of each HDL subfraction and plasma apoA-I level that was measured by turbidity immunoassay (ApoAuto AI, Daiichi Pure Chemicals). The reproducibility of the native 2D-gel technique has been described in our previous publications (14, 21, 25–28).

Statistical analyses

We used StatView (version 5.0J) for statistical analyses. All values were expressed as mean ± SE. Changes in an individual subject were analyzed using Student's paired t-test. The correlation between the two methods was evaluated using Pearson's correlation coefficient. We considered the difference between two groups to be statistically significant if the P value was less than 0.05.

RESULTS

Effects of storage temperature and duration on preβ1-HDL concentration

Storage temperature affected the measured preβ1-HDL concentration in the normolipidemic and hyperlipidemic subjects. At room temperature (24°C), the measured preβ1-HDL concentration decreased as a function of time (**Fig. 1**, open and closed squares), whereas at 4°C, the measured preβ1-HDL concentration increased slowly with time (Fig. 1, open and closed circles). When plasma was stored on ice water, however, no change in apparent preβ1-HDL concentration was observed for at least 4 h (Fig. 1, open and closed triangles).

Neither an LCAT inhibitor nor blood cells were observed to stabilize the concentration of preβ1-HDL at room temperature. When DTNB was added to plasma, the preβ1-HDL concentration did not decrease, but rather increased significantly during storage (**Fig. 2**, open and closed circles). When samples were stored as whole blood (Fig. 2, open and closed squares), a decrease in measured preβ1-HDL concentrations occurred at nearly the same rate as for stored plasma samples (Fig. 2, open and closed triangles).
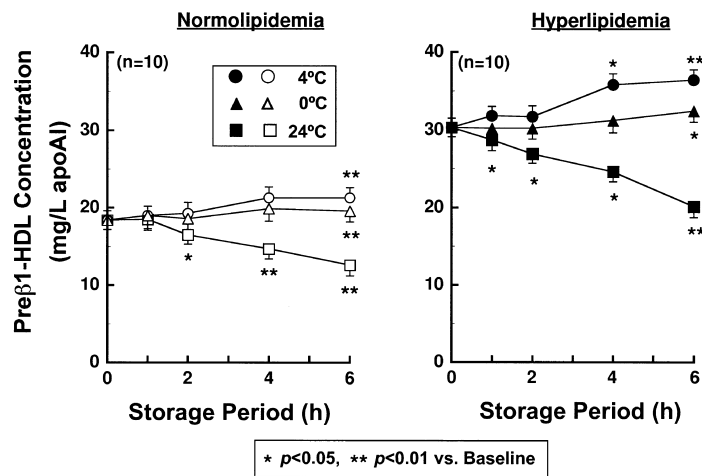
Fig. 1. Changes in preβ1-HDL concentration during storage at different temperatures. Plasma was obtained from 10 normo-lipidemic (open symbols) and 10 hyperlipidemic volunteers (closed symbols: hypercholesterolemia, n = 4; hypertriglyceri-demia, n = 3; combined hyperlipidemia; n = 3). Plasma was stored at the indicated temperatures for 6 h. Samples were taken at intervals, and preβ1-HDL concentrations were imme-diately determined at each sampling point by immunoassay as described in Materials and Methods.

A sucrose-stabilizing buffer completely inhibited changes in measured preβ1-HDL concentration during storage in both normolipidemic and hyperlipidemic subjects. As de-scribed above, samples stored at 4°C without pretreatment exhibited increases in preβ1-HDL, so that the mean preβ1-HDL concentration at day 5 was almost double the baseline level (**Fig. 3**, open and closed circles). Although preβ1-HDL levels appeared stable for 4 h when samples were stored on ice water, preβ1-HDL levels did increase over longer storage periods (Fig. 3, open and closed trian-gles). In contrast, samples stored at 4°C after pretreat-ment exhibited no change in preβ1-HDL concentration for 5 days (Fig. 3, open and closed squares).

Effect of freezing on measured preβ1-HDL concentration

When nonpretreated samples were stored at −80°C for 30 days, the mean preβ1-HDL concentration increased significantly in the hyperlipidemic subjects (**Fig. 4**, right open and hatched bars). However, preβ1-HDL did not in-crease significantly in the normolipidemic subjects (Fig. 4, left open and hatched bars). In contrast, no samples that were pretreated prior to freezing exhibited a significant change from baseline levels (Fig. 4, closed bars). We also tested samples that were stored at −20°C for 30 days. In

these samples, the measured preβ1-HDL concentrations of fresh and frozen samples did not differ significantly (n = 6; 94.8 ± 7.0% of the baseline level).

Comparison of sandwich enzyme immunoassay and native 2D-gel electrophoresis techniques

In this comparison, the outpatient samples were used, because since they exhibited a broader range of preβ1-HDL concentrations than did those from the healthy vol-unteers, and were thus better suited to determining the applicability of these techniques to a clinical situation. Preβ1-HDL concentrations determined by immunoassay using pretreated samples had strong positive correlations with those determined by native 2D-gel electrophoresis us-ing fresh plasma (**Fig. 5**). However, values determined by immunoassay were lower than those determined by native 2D-gel electrophoresis.

DISCUSSION

These results clearly indicate that our immunoassay technique using MAb55201 with pretreated samples is a precise and reproducible method for preβ1-HDL mea-
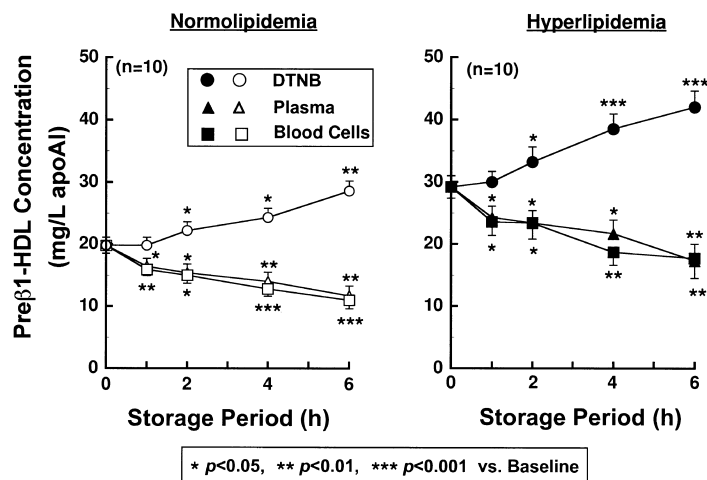

Fig. 2. Effect of 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) and blood cells on preβ1-HDL stability during storage at room temperature. Blood was drawn from 10 normolipidemic (open symbols) and 10 hyperlipidemic volunteers (closed symbols: hy-percholesterolemia, n = 4; hypertriglyceridemia, n = 3; com-bined hyperlipidemia, n = 3). Samples of plasma alone (trian-gles), plasma with DTNB (2 mmol/l, circles), and plasma with blood cells (squares) were stored at room temperature (24°C) for 6 h. Samples were taken at intervals, and preβ1-HDL con-centrations were immediately determined at each sampling point by immunoassay.
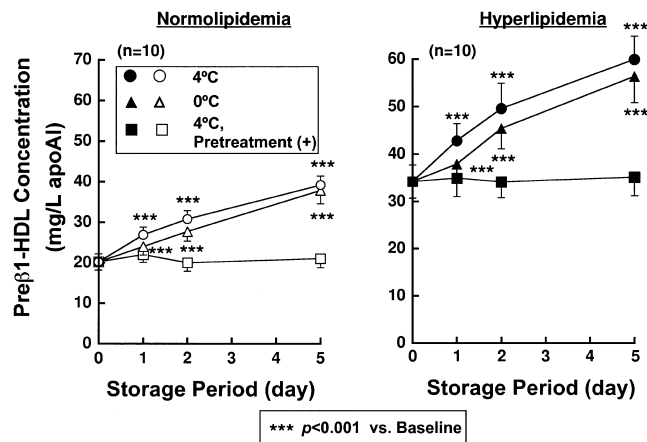
Fig. 3. Effect of pretreatment on preβ1-HDL stability during storage. Plasma was obtained from 10 normolipidemic (open symbols), and 10 hyperlipidemic volunteers (closed symbols: hypercholesterolemia, n = 4; hypertriglyceridemia, n = 3; combined hyperlipidemia; n = 3). Plasma was stored at 0°C without pretreatment (triangles; 1:20 dilution (v/v) with 50% sucrose), and at 4°C with (squares) or without pretreatment (circles) for 5 days. Samples were taken at intervals, and preβ1-HDL concentrations were immediately determined on each sampling day by immunoassay.

surement in plasma samples. We found that preβ1-HDL in pretreated samples was stable for up to 5 days at 4°C (Fig. 3) and for up to 30 days at −80°C (Fig. 4) in both normolipidemic and hyperlipidemic subjects. Moreover, the preβ1-HDL levels determined by immunoassay correlated significantly with those determined by native 2D-gel electrophoresis (Fig. 5), which is the generally accepted method for assaying preβ1-HDL (11, 14, 21, 23–28).

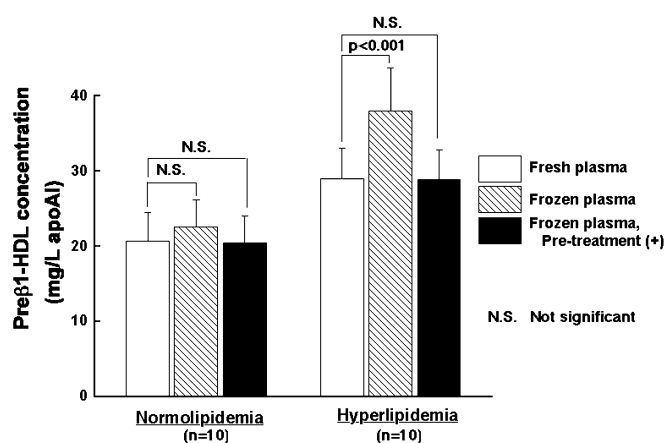Accurate measurement of preβ1-HDL levels in stored samples requires awareness of the possibility of intercon-

Fig. 4. Effect of pretreatment on preβ1-HDL stability during storage at –80°C. Plasma was obtained from 10 normolipidemic (left bars), and 10 hyperlipidemic volunteers (right bars: hypercholesterolemia, n = 4; hypertriglyceridemia, n = 3; combined hyperlipidemia; n = 3) Plasma was frozen with (closed bars) or without pretreatment (hatched bars), and stored at –80°C. The baseline preβ1-HDL concentrations in fresh plasma (open bars) were measured by immunoassay on the same day that blood was obtained. The frozen samples were thawed 30 days later, and preβ1-HDL concentrations were determined by immunoassay.
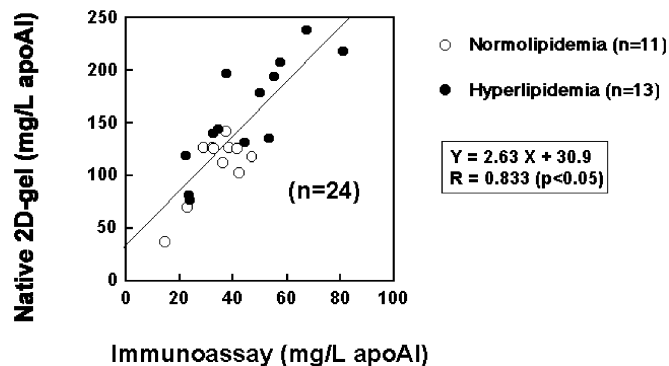

Fig. 5. Comparison of enzyme immunoassay with native 2D-gel electrophoresis as an analytical technique for measuring preβ1-HDL concentrations in plasma samples. Plasma was obtained from 24 outpatients (normolipidemic, n = 11; hypercholesterolemic, n = 6; hypertriglyceridemic, n = 3; combined hyperlipidemic, n = 4). Preβ1-HDL concentrations were measured by immunoassay (using sucrose pretreated samples) and native 2D-gel electrophoresis (using fresh plasma). For immunoassays, pretreated samples were stored at 0°C, and preβ1-HDL concentrations were determined within 4 h of sample donation.

version of preβ1-HDL and α-HDL during storage. The conversion of preβ1-HDL to α-HDL is promoted by LCAT (15, 28), whereas the reverse reaction is promoted by many factors, including CETP (21, 29), PLTP (30–31), hepatic lipase (27, 32), and serum amyloid A protein (33–34). During a 90 min incubation at 37°C, about 80% of preβ1-HDL is converted into α-HDL in normolipidemic subjects (28). This phenomenon can be blocked by inhibition of LCAT or by coincubation with certain types of cells, such as fibroblasts and macrophages (28). In our previous study, blood cells did not prevent preβ1-HDL from converting to α-HDL (28); indeed, the apparent preβ1-HDL concentrations in whole blood and plasma decreased by similar amounts when stored at room temperature (Fig. 2).

Storage of plasma at 4°C reduces the rate at which preβ1-HDL is converted to α-HDL (Fig. 1). However, apparent preβ1-HDL levels increased gradually for samples stored at 4°C for long periods (Figs. 1, 3). This finding strongly suggests that preβ1-HDL is generated from α-HDL under these conditions. Jaari et al (31) transiently overexpressed human PLTP in mice by an adenovirus-mediated method, and then incubated plasma from these mice at 37°C with an LCAT inhibitor (iodo-acetate). They found that total preβ-HDL in these samples increased as a function of time, and that PLTP activity was positively correlated with the ability to generate preβ-HDL during the 37°C incubation. Therefore, preβ1-HDL may be generated by PLTP activity during long storage at 4°C. PLTP activity may be also involved in the increase in preβ1-HDL in the presence of DTNB (Fig. 2).

When pretreated with 50% sucrose, preβ1-HDL is very stable during storage in both normolipidemic and hyperlipidemic subjects. In pretreated samples, preβ1-HDL levels did not change at all for 5 days at 4°C (Fig. 3). Moreover, pretreated samples can be frozen and stored for up

to 30 days at either $-20°C$ or $-80°C$ with no apparent effect on preβ1-HDL levels (Fig. 4). In our experiments, preβ1-HDL levels in frozen samples from normolipidemic subjects did not change significantly over time, but preβ1-HDL levels in frozen samples from hyperlipidemic subjects increased significantly over time. We have tested many reagents, including protamine, protamine sulfate, sodium fluoride, phenylmethylsulfonylfluoride, aprotinin, benzamidine, sodium azide, and surfactants for their ability to stabilize preβ1-HDL. However, none of these treatments worked as well as the 50% sucrose solution (data not shown). Pretreatment with sucrose solution is an inexpensive, simple, and reliable method. Since pretreated samples are stable at $-20°C$, samples can easily be stored in a $-20°C$ freezer until assayed.

The immunoassay with pretreated samples offers other advantages over the native 2D-gel electrophoresis method for measuring preβ1-HDL. First, the immunoassay requires much less time to complete. According to our protocol (14, 21, 25–27, 33), native 2D-gel electrophoresis takes about 1 day (2 h and 20 h for agarose and gradient polyacrylamide gel electrophoresis, respectively), electrophoretic transfer and immunoblotting take another day, and exposure to X-ray film takes another 1 to 2 days. In contrast, the immunoassay can be completed within 3 h. Second, the immunoassay does not require special technical skills, significant space, or use of radioisotopes. Finally, many samples can be measured simultaneously by the immunoassay technique, whereas only four samples can be analyzed in one 2D-gel run.

It should be noted that preβ1-HDL concentrations as determined by immunoassay were decreased by almost two thirds compared with those determined by native 2D-gel electrophoresis (Fig. 5). Such discrepancy may result from two possible reasons, that is, underestimation of apoA-I of preβ1-HDL by immunoassay or overestimation of apoA-I of preβ1-HDL by native 2D-gel electrophoresis. In our previous study, we added MAb55201 to fresh plasma, and kept it on ice for 5 min. In the analysis using native 2D-gel electrophoresis, all plasma preβ1-HDLs reacted with MAb55201 to form preβ1-HDL/antibody complexes (15). As described in Materials and Methods, MAb55201 measures most apoA-I of the small HDL subfraction as preβ1-HDL. Although preβ1-HDL may expose less epitopes of apoA-I than delipidated apoA-I, insufficient reactivity of MAb55201 is not likely to be the main cause of such a big difference between immunoassay and 2D-gel electrophoresis.

Determination of preβ1-HDL concentrations by native 2D-gel is highly dependent on the techniques and antibodies used for the assays. In fact, in the earlier studies of normal subjects, 2D-gel determination of mean preβ1-HDL concentrations yielded values that varied from $1.3 \pm 0.8\%$ to $9.2 \pm 3.1\%$ of plasma apoA-I values (11, 14, 21, 23), demonstrating that results from 2D-gel are highly variable. Among institutions measuring preβ1-HDL concentrations, conditions for 2D-gel electrophoresis are not really standardized. For example, the electrophoresis temperature and time for the 2D-gel range from 0 to 10°C,

and from 1.5 to 20 h. As we have shown in the present study, plasma preβ1-HDL concentration increases steadily, even at 0°C (Figs. 1, 3). These results suggest that significant amounts of preβ1-HDL may be newly generated during native 2D-gel electrophoresis. The mechanical friction through the gradient polyacrylamide gels might cause some dissociation of preβ1-HDL from α-HDL. In addition, it is conceivable that the relative concentrations of minor HDL subfractions are somewhat overestimated due to their background radioactivities. In γ spectrometry, we cannot avoid measuring background counts around the spots of HDL subfractions. The ratio of background to proper count must be higher in minor HDL subfractions than in major HDL subfractions. The high ratio probably results in the increase in preβ1-HDL concentration determined by native 2D-gel electrophoresis. Thus, the MAb55201-based ELISA may be more suitable than electrophoresis for standardization of preβ1-HDL concentrations.

In summary, an immunoassay using MAb55201 with pretreated samples is a precise and reproducible method for measuring preβ1-HDL levels in plasma samples. This immunoassay may be used clinically to aid in detection of subjects at risk for atherosclerotic disorders. 🔳

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