procedure to obtain lymphoblast extracts produced more concentrated enzyme extracts with higher specific activities.

By performing the assay in the presence of various concentrations of ornithine, we confirmed that AGAT is inhibited by ornithine, as reported previously (7). This observation may be of clinical interest in conditions of hyperornithinemia. Decreased brain creatine has been observed in patients affected by gyrate atrophy of the choroid and retina (McKusick 258870). It has been suggested that in this condition the high concentrations of ornithine caused by the inherited deficiency of ornithine-δ-aminotransferase inhibit AGAT and, thus, creatine biosynthesis (8). In addition, in hyperornithinemia-hyperammonemia-homocitrullinuria syndrome, creatine excretion has been shown to be low (9).

In our in vitro assay, the amount of ornithine formed posed no problem. Inhibition became significant at an ornithine concentration of ~0.1 mmol/L (93% remaining activity), whereas during incubations over 20 h, at most 3 nmols of ornithine were formed (0.015 mmol/L).

It is known that creatine regulates AGAT at the pre-translational level. We investigated whether creatine also inhibits AGAT activity directly. We observed no inhibition of AGAT by creatine at concentrations up to 5 mmol/L.

In conclusion, we present a sensitive and specific enzymatic assay for AGAT that enables enzymatic diagnosis of AGAT deficiency. It may help in the diagnosis of more patients with AGAT deficiency. Early recognition and treatment may effectively prevent neurologic damage (1).

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References


Identification and Properties of Glycated Monoclonal IgA That Affect the Fructosamine Assay, Kiyotaka Fujita,1* Linda K. Curtiss,2 Ikunosuke Sakurabayashi,2 Funiko Kameko,3 Nobuo Okumura,3 Funiko Terasaow,5 Minoru Totsuka,4 and Tsutomu Katayama4 (1) Department of Immunology, The Scripps Research Institute, 10550 North Torrey Pines Rd., La Jolla, CA 92037; (2) Department of Clinical Laboratories, Omiya Medical Center, Jichi Medical School, Amanuma, Saitama 330-8503, Japan; (3) Department of Biomedical Laboratory Sciences, School of Health Sciences, Shinshu University, Asahi, Matsumoto, Nagano 390-8621, Japan; (4) Department of Laboratory Medicine, Shinshu University School of Medicine, Asahi, Matsumoto, Nagano 390-8621, Japan; *author for correspondence: fax 858-784-9144, e-mail kyfujit@aol.com

Serum fructosamine is used to monitor short-term (1–3 weeks) average glycemia (1, 2), but controversy remains whether it is influenced by the serum concentrations of albumin (3, 4), IgA (5, 6), or monoclonal IgA (7, 8). We recently demonstrated glycation of monoclonal IgA and the presence of IgA-albumin complexes (9), but the relationship between the glycation of monoclonal IgA and the IgA-albumin complexes was not clear.

In this study, we measured immunoglobulin-albumin complexes, serum fructosamine, and the glycation of immunoglobulin in patients with monoclonal proteinemia and in patients with polyclonal hyper-IgA.

We obtained serum samples from 40 patients with M-proteinemia with no history of diabetes and whose plasma glucose concentrations were <1.1 g/L. The M-protein was IgG in 17 of the patients [mean (SD), 29.2 (21.0) g/L], IgA in 13 [18.5 (16.4) g/L], and IgM in 10 [16.8 (11.3) g/L]. We also analyzed sera from 15 nondiabetic patients with hepatitis who had polyclonal hyper-IgA [5.1 (1.1) g/L] as controls.

Serum fructosamine was measured at 37 °C in an automated analyzer (JCA-RX 10 Clinalyzer; Japan Electron Optics Laboratory) with reagents from Roche Diagnostics Corporation.

Serum protein electrophoresis was performed on agarose gels. Immunoelectrophoresis was based on the method of Scheidegger (10). Fructosamine was detected on agarose gels by incubating the gel for 30 min at 37 °C with a 2X concentration of a fructosamine reagent.

IgA was isolated from serum with use of a jacalin-agarose (Funakoshi Corporation) column (11) and 0.8 mol/L galactose to elute the IgA. The IgA fraction was dialyzed against 0.2 mol/L Tris-HCl buffer (pH 8.0), concentrated by ultrafiltration, loaded on a DEAE-Sepharose (Amersham Pharmacia Biotech) ion-exchange column, and eluted with a linear gradient of 0–0.5 mol/L NaCl in the 0.2 mol/L Tris-HCl buffer.

Sera were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), with or without 2-mercaptoethanol (2-ME), and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (12). Immunostaining was performed with rabbit polyclonal antiserum (Dako) as first antibodies and peroxy-
dase-conjugated goat anti-rabbit IgG (Dako) as second antibody.

To detect fructosamine on PVDF membranes, we blocked the membranes for 60 min at room temperature in buffer containing 30 g/L bovine serum albumin. After washing, the membranes were incubated for 30 min at 37°C with a 2× concentration of the fructosamine reagent.

Differences between the groups were verified by the Student t-test. Correlations were studied by means of the Pearson correlation coefficient.

Serum fructosamine concentrations were higher in patients with IgA-type [3.47 (0.93) mmol/L; P <0.05] than with IgG-type [2.55 (0.48) mmol/L] or IgM-type M-proteinemia [2.51 (0.42) mmol/L]. Concentrations of fructosamine and of serum IgA (but not IgG or IgM) in patients with M-proteinemia were significantly correlated (γ = 0.698; P <0.05). By contrast, in the 15 patients with hepatitis and polyclonal hyper-IgA, mean (SD) fructosamine was 2.62 (0.23) mmol/L and all values were within our reference interval (1.94–3.16 mmol/L); in addition, fructosamine was not significantly correlated with the IgA concentrations (γ = 0.279).

The sera from the patients with IgG- or IgM-type M-proteinemia had fructosamine only at the position of albumin, but 11 of 13 sera with IgA-type M-proteinemia stained for glycoprotein at the position of the M-protein band as well as the albumin band (Fig. 1 in the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol49/issue5/). Sera from the patients with polyclonal hyper-IgA did not stain for glycoproteins at the position of IgA but did stain at the position of the albumin band.

IgA-κ-type M-protein also reacted with albumin-specific antisera (Fig. 1). The same abnormal precipitin arcs were observed in 11 of 13 sera from patients with IgA-type M-proteinemia that were glycosylated at the position of M-protein band. Moreover, the mobility of the abnormal precipitin arcs corresponded to the position of the glycosylated M-protein band. The patients’ albumin had a macromolecular mass >300 kDa, as determined by immunostaining using anti-albumin serum after SDS-PAGE without 2-ME.

The abnormal precipitin arcs for albumin disappeared when antisera specific for human albumin or IgA (α-chain specific) were incubated for 37°C for 30 min with the purified IgA-type M-protein (at a ratio of 6:1 by volume) and supernates were concentrated by ultrafiltration. The immunoelectrophoretic patterns of the sera from patients with IgG- or IgM-type M-proteinemia or with polyclonal hyper-IgA showed no abnormal precipitin arcs with albumin.

After SDS-PAGE with 2-ME, macroalbumin was not detected by immunostaining with anti-albumin serum, and IgA-type M-proteins stained for fructosamine at ~55 kDa, which corresponded to the α-chain, and also at 25 kDa, which corresponded to the light chain, in 11 of 13 cases. Sera from patients with IgG- or IgM-type M-proteinemia or with polyclonal hyper-IgA showed fructosamine glycosylation only at 65 kDa, which corresponded to albumin (Fig. 2 in the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol49/issue5/).

To investigate the possibility that monoclonal IgA was glycosylated in the presence of glucose, we incubated a patient’s serum that had no fructosamine glycosylation at the M-protein band with d-glucose at 10 or 20 g/L. Incubation of the monoclonal IgA serum with 10 or 20 g/L glucose at 37°C for 60 min produced no fructosamine at the position of the monoclonal IgA band on electrophoresis, but increased the fructosamine at the position of albumin band (Fig. 3 in the Data Supplement).

Glycohemoglobin is an accepted measure of long-term average glycemia. Because nonenzymatic glycation also affects other proteins (13), glycated albumin or total glycated protein (fructosamine assay) can be used as a measure of short-term, average glycemia (1, 2). Wahid et al. (14) stated that serum fructosamine is useful as a maker of 5-year risk of developing diabetes mellitus in patients exhibiting stress hyperglycemia; however, the dependence of fructosamine concentrations on the concentrations of albumin and total protein has been reported (15–19). Montagna et al. (7) and Nakamura et al. (8) reported that nondiabetic patients with IgA-type M-proteinemia have significantly increased serum fructosamine.

![Fig. 1. Electrophoretic (A) and immunoelectrophoretic (B) patterns of a serum from a patient with IgA-κ-type M-proteinemia.](image-url)
We also found that the fructosamine concentrations in patients with IgA-type M-proteinemia were significantly higher than the fructosamine concentrations in patients with IgG- or IgM-type M-proteinemia. By staining for fructosamine after electrophoresis, we demonstrated that this is the result of an increase in glycated monoclonal IgA, i.e., 84.6% of the patients with IgA-type M-proteinemia had increased fructosamine at the monoclonal IgA bands, and fructosamine was detected in both the heavy and light chains.

The two patients with IgA-type M-proteinemia who did not have fructosamine at the M-protein band had lower IgA values than did the 11 patients with IgA-type M-proteinemia. This suggests that glycation of IgA molecules may be related to the high IgA concentrations [mean (SD), 18.5 (16.4) g/L]. The sera from the patients with polyclonal hyper-IgA had fructosamine only in the albumin band. Thus, glycation of IgA molecules appears to be unique to monoclonal IgA.

It is interesting that the macroglobulin complexes that were composed of IgA and albumin were detected in all sera from patients with IgA-type M-protein containing fructosamine at the M-protein band (11 of 13). The IgA-albumin complex was absent from the sera from patients with IgG- or IgM-type M-proteinemia as well as from the sera from patients with polyclonal hyper-IgA. Moreover, monoclonal IgA that was not associated with albumin was not glycosylated, although the glucose concentration in the patient’s serum was increased. Thus, a major factor in the glycation of IgA was the binding to albumin. Because the half-life of albumin is longer (15–19 days) (20) than the half-life of human IgA molecules (6 days) (21), the half-life of monoclonal IgA that binds albumin is longer, and this could increase the glycation of the monoclonal IgA.

Why is the monoclonal IgA glycosylated and not the polyclonal IgA? The mean IgA concentrations (18.5 g/L) in patients with monoclonal IgA were clearly higher than the IgA concentrations (5.1 g/L) in patients with polyclonal IgA. Among the patients with IgA concentrations <10 g/L, 3 of the 5 patients with monoclonal IgA had IgA-albumin complexes, but none of the 15 patients with polyclonal IgA had IgA-albumin complexes (Fig. 4 in the Data Supplement). Moreover, serum IgA is present in several polymeric forms, ranging from monomers to pentamers. In normal human serum, the monomeric form usually predominates, but in monoclonal IgA serum, the relative amount of each polymeric form varies. Polymerization of the monoclonal IgA might therefore play an important role in its interaction with albumin. This idea is supported by the fact that the IgA-albumin complexes had a macromolecular mass >300 kDa.

The formation of IgA-albumin complexes has been noted previously (22–25). Tomasi and Hauptsman (23) examined 49 IgA myeloma sera and found that 65% had IgA-albumin complexes, suggesting that albumin was bound predominantly to dimeric IgA.

Why is the abnormal precipitin arc for albumin faster than that for IgA on immunoelectrophoresis? Serum protein electrophoresis usually separates the various components of blood protein into bands or zones according to their electrical charges. The isoelectric points of immunoglobulins are 6.4–7.2, whereas that of albumin is 4.8 (26). It can be considered that the abnormal precipitin arc for albumin in the IgA-albumin complexes moves faster than that for IgA because of the acidity of the albumin.

In conclusion, our findings throw doubt on the clinical utility of fructosamine as a measure of hyperglycemic status in patients with IgA-type M-proteinemia.

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References

Fully Automated Chemiluminometric Assay for Hyperglycosylated Human Chorionic Gonadotropin (Invasive Trophoblast Antigen), Raj Pandian, Julie Lu, and Jolanta Ossolinska-Plewnia

Hyperglycosylated human chorionic gonadotropin (HhCG) is a glycoprotein hormone secreted during embryonic implantation and trophoblast invasion of the uterine wall and is an early marker of pregnancy (1). Relative to hCG, HhCG has a higher molecular mass (38.5–40 kDa, depending on the amount of carbohydrate) and a higher number of asparagine (N)-linked triantennary carbohydrates and serine (O)-linked tetrasaccharide core structures in the β-subunit (2). Although both are secreted from the placenta and choriocarcinoma, HhCG is produced by mononucleated cytotrophoblasts, and hCG is produced by syncytiotrophoblast cells (3–6). Because the cytotrophoblasts are primitive and invasive in nature, HhCG is also called invasive trophoblast antigen (ITA) (5).

Birken et al. (7) described a monoclonal antibody (B152) specific for the β-subunit C-terminal peptide and the O-linked oligosaccharide of HhCG. Although the epitope for this antibody does not require sialic acid, the presence of the O-linked tetrasaccharide core structure is essential (1).

Using IRMAs and ELISAs, investigators showed that (a) HhCG rapidly increases in early pregnancy, attaining substantially higher concentrations and decreasing earlier than hCG (1, 8); (b) HhCG is increased in Down syndrome-affected pregnancies in both the first and second trimesters (9–11); and (c) the HhCG:hCG ratio appears to be higher in those with invasive vs noninvasive trophoblastic disease (12).

The above HhCG assays were performed manually using large sample volumes (200 µL) and long incubation times (turnaround time, 1–2 days). We therefore developed an automated immunochemiluminometric assay (ICMA) that uses two monoclonal antibodies: the HhCG-specific B152 antibody described above and a hCG β-subunit-specific antibody (B207). Both antibodies were purified from cell lines provided by Dr. O’Connor (Columbia University, New York, NY). B152 was biotinylated with long-chain NHS-biotin (13), and B207 was conjugated with acridinium ester (14).

The Nichols Institute Diagnostics Advantage instrument automatically pipetted 15 µL of sample into a cuvette, followed by 25 µL of streptavidin-coated magnetic particles (4 g/L Dynal M-270), 70 µL of capture antibody (6 mg/L B152), and 260 µL of buffer [0.1 mol/L phosphate-buffered saline (PBS), pH 8.2, containing 50 g/L bovine serum albumin (BSA)]. During a 30-min incubation at 37 °C, HhCG in the sample bound to the B152 capture antibody, which in turn bound to the magnetic particles. The magnetic particles were automatically washed three times to remove unbound materials. Detection antibody [300 µL of 1 mg/L B207 in 0.5 mol/L PBS (pH 7.4) with 5 g/L protease-free BSA, 60 mL/L normal mouse serum, and 1 g/L mouse γ-globulin] was then added to the washed magnetic particles. During this 10-min incubation at 37 °C, the B207 antibody bound to a hCG-shared epitope on the HhCG molecule, forming a sandwich complex. After another three washes, the magnetic particle-containing wells were transferred to the on-board luminometer. Hydrogen peroxide (3.25 mL/L) and sodium hydroxide (0.25 mol/L)-containing solutions were automatically injected into the wells, initiating the chemiluminescence reaction. The generated “flash” of light was quantified and expressed as relative light units (RLU). The RLU are directly proportional to the concentration of HhCG in the sample. The relationship was linear up to 7500 µg/L, at which concentration the curve plateaued (Fig. 1). No hook effect was observed with concentrations as high as 30 000 µg/L [HhCG from choriocarcinoma, prepared in 0.5 mol/L PBS (pH 7.4) containing 1 g/L protease-free BSA].

The calibration curve (Fig. 1, inset) was stored by the instrument, so calibrators did not have to be included every time the assay was performed. The limit of detection, based on the mean RLU for 20 replicates of the zero calibrator plus 2 SD, was 0.1 µg/L. The limit of quantification, based on assays of serial dilutions of a sample (HhCG = 2 µg/L) with the CV calculated from 10 observations at each dilution, was 0.2 µg/L (CV <20%). On the basis of results from three serum pools and one urine pool, the intraassay CV for 20 replicates was <3.5%, and the interassay CV was <7.5%. Thus, the assay could be performed in singlicate.

The assay had <0.1% cross-reactivity with all glycoprotein hormones except hCG, which had a cross-reactivity <3.5% (Table 1). The dose-response of each hCG preparation (total, free β, nicked, and nicked free β-hCG) was parallel to that of HhCG. Although hCG cross-reactivity was minimal, it is not clear whether such cross-reactivity was attributable to native hCG itself or to contamination of hCG preparations with HhCG. The latter is highly likely because of (a) the different cross-reactivities reported for various hCG preparations [Table 1 and Refs. (5, 7)]; (b) the similarities between dose-response curves for HhCG and the cross-reactants; and (c) the 0.9% cross-reactivity observed when recombinant murine hCG was tested (Table 1). Because the reported hCG cross-reactivity was small, HhCG could be measured in the presence of hCG. The HhCG:hCG ratio may be clinically useful (1, 15, 16).

To determine the suitability of various sample types,