Time-Resolved Immunofluorometric Assay of 34-kDa Somatomedin-Binding Protein

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In this time-resolved immunofluorometric assay for the 34-kDa somatomedin-binding protein (SmBP), affinity-purified polyclonal antibodies are used, along with solid-phase separation of bound and free analyte. The first antibody is bound to polystyrene microtiter wells; the second is labeled with europium(III) chelate. The detection limit of the method is 0.25 μg/L, much lower than that (about 8 μg/L) for radioimmunoassay. By immunofluorometric assay, SmBP is detectable, and could be accurately quantified, in the serum of all 88 individuals we tested, whereas by radioimmunoassay a third of the samples had concentrations below the detection limit. When SmBP was detectable by both methods, the concentrations measured by the two techniques correlated well (r = 0.98).

Additional Keyphrases: pregnancy • hepatoma • amniotic fluid • radioimmunoassay compared • insulin-like growth factors

Somatomedins, or insulin-like growth factors, are bound to specific binding proteins in blood. Two forms of binding proteins, with molecular masses of 150 kDa and about 40 kDa, have been identified in human serum (1, 2). Most of the circulating somatomedin-C is bound to the 150-kDa binding protein. Human amniotic fluid contains another SmBP, with a molecular mass of 34 kDa (3), that is identical to placental protein 12 (PP12), as evidenced by similar somatomedin-binding properties and identical N-terminal amino acid sequences (4). The 34-kDa SmBP is synthesized by secretory/dedifferentiated endometrium (5, 6). A human hepatoma cell line also produces an SmBP identical to the one isolated from amniotic fluid (7). Radioimmunoassays (RIAs) developed for the 34-kDa SmBP (8–10) readily detect the protein in sera in pregnancy from the eighth week onwards (8). In nonpregnant women and especially in men, however, the concentration of 34-kDa SmBP is often below the detection limit of currently available RIAs (8). Changes in the circulating concentrations of SmBPs have been observed in various clinical conditions (11, 12), but in the absence of a sensitive method it has not been possible to assess the significance of low concentrations.

Time-resolved immunofluorometric assay (IFMA) is a new highly sensitive assay technique (13). We report here the development of an IFMA for the 34-kDa SmBP that is 30 times more sensitive than RIA and allows quantification of this protein in all serum samples from men and nonpregnant women.

Materials and Methods

SmBP and its antibody. The 34-kDa SmBP was purified from amniotic fluid by gel filtration, hydrophobic interaction, and ion-exchange chromatography (14). SmBP-containing fractions from gel filtration were applied to a hydrophobic-interaction column (TSKgel Phenyl-5PW; Toyo Soda, Tokyo, Japan) that was equilibrated with 0.2 mol/L sodium phosphate, pH 7.1. SmBP was eluted by decreasing the concentration of sodium phosphate to 0.01 mol/L and increasing the concentration of isopropanol from 0 to 50 mL/L during 30 min. Material corresponding to the main peak showed only one band in sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and it was used for immunizations. A rabbit was injected subcutaneously with purified SmBP at three-week intervals. The first two injections contained 190 μg of purified SmBP emulsified in Freund’s complete adjuvant (Difco Laboratories, Detroit, MI). Booster injections contained first 190 μg and later 60 μg of SmBP in incomplete Freund’s adjuvant. The first blood specimen was collected two weeks after the fourth immunization.

We coupled purified SmBP to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) according to the manufacturer’s instructions, then purified the anti-SmBP immunoglobulin from the rabbit by affinity chromatography on the SmBP-Sepharose column. The bound antibody was eluted from the column with 2 mol/L KSCN reagent, then dialyzed against Na2BO3 (0.1 mol/L, pH 9.0).

Immobilization of anti-SmBP antibodies. Anti-SmBP immunoglobulin was adsorbed onto polystyrene microtiter wells (Eflab Oy, Helsinki, Finland) by incubation overnight at 4 °C. We used, per well, 200 μL of a 5 mg/L solution of purified antibody in Tris HCl buffer (150 mmol/L, pH 7.7) containing 9 g of NaCl and 0.5 g of NaBr per liter ("Tris buffer"). After washing the strips with wash solution (9 g of NaCl, 0.5 mL of Tween 40, and 0.5 g of NaBr per liter) we saturated them 1 h at room temperature with 250 μL of a 10 g/L solution of bovine serum albumin in Tris buffer. After another wash we stored the strips in a moist atmosphere at 4 °C. The purified SmBP-antibody was labeled with isothiocyanatophenyl-EDTA-europium(III) as described before (15).

Immunofluorometric assay (IFMA). Add 25 μL of standard or sample, in duplicate, and 200 μL of assay buffer (Tris buffer containing 5 g of bovine serum albumin, 0.5 g of bovine IgG, and 0.1 mL of Tween 40 per liter) to the coated microtiter wells and incubate for 2 h at room temperature, or overnight at 4 °C. Then wash the wells five times with wash solution, add to each well 100 ng of Eu-labeled antibody in 200 μL of assay buffer, and incubate at room temperature for 1 h. Again, wash five times, then add 200 μL of enhancement solution consisting of 0.1 mL of Triton X-100, 15 μmol of 2-naphthyltrifluoracette, and 50 μmol of tri-n-octylphosphate oxide per liter of 0.1 mol/L acetate-phthlate buffer, pH 3.2 (LKB Wallac, Turku, Finland). Shake the microtiter wells gently, and, after 5 min, measure the fluorescence. We used a 1230 Arcus fluorometer (LKB Wallac) counting for 1 s (13). The RIA was performed as described earlier (8). SmBP was iodinated with Na125I by use of the Chloramine-T method. Antiserum to PPI2 (lot no. 461ZA; Behringwerke AG, Marburg, F.R.G.) was diluted 1:4000 in sodium phosphate buffer (50 mmol/L, pH 7.4) containing 9 g of NaCl and 10 g of bovine serum albumin.
per liter. We incubated 100 µL each of standard, tracer, and antiserum overnight and separated the antigen–antibody complex by using 50 µL of solid-phase second antibody (Sac Cell; Wellcome, Beckenham, U.K.). We used the RIA and the IFMA to determine concentrations of SmBP in serum from nonpregnant women and men. As a standard we used calibrated amniotic fluid, i.e., fluid in which the SmBP concentration had previously been estimated by RIA. For the comparison with IFMA, we used 50-µL samples in the RIA, to reduce nonspecific effects.

Results

The measuring range of the IFMA was 0.25–250 µg/L, with a nearly linear dose–response curve over the whole range. Parallel dose–response curves were observed with purified SmBP (0.25–250 µg/L), amniotic fluid (diluted 200-fold to 51 200-fold), pregnancy serum (undiluted to 64-fold diluted), and plasma from healthy, nonpregnant adults (undiluted to eightfold diluted) (Figure 1). The following human proteins showed less than 0.05% cross reactivity by weight: human placental lactogen, pregnancy-specific β1-glycoprotein, alpha-fetoprotein, placental protein 10 (Behringwerke AG), placentin (Behringwerke AG), and prolactin. The detection limit of the assay was calculated to be 0.25 µg/L, this limit being defined as the SmBP concentration corresponding to the mean fluorescence signal for 12 replicates of a zero sample (25 µL of 10 g/L bovine serum albumin) plus three standard deviations (1021 ± 247 counts/µL). The intra- and interassay variation was <10% at SmBP concentrations between 1.2 and 92 µg/L (Table 1). Analytical recovery of SmBP added to serum samples containing 1.2 and 14.1 µg of endogenous SmBP per liter was in the range of 90 to 105% (Table 2).

Concentrations of SmBP in serum from 46 pregnant women and 42 men as determined by RIA (y) and by IFMA (x) correlated well: y = 0.91x + 8.8 µg/L, r = 0.98. For the nonpregnant women the median concentration as determined by IFMA was 25.1 (range 1.3–95) µg/L; by RIA, the median was 31 (<19.2–98) µg/L. For men, the median concentration as determined by IFMA was 10.3 (2.2–84) µg/L; the range determined by RIA was <19.2–95 µg/L, the median being below the detection limit (<19.1 µg/L).

Discussion

This immunofluorometric method is 30-fold as sensitive as RIA, permitting accurate quantification of the 34-kDa SmBP in serum from all the subjects we studied. The sample volume required is only 25 µL and total incubation time is 3 h, as compared with 100 µL and 18 h for the RIA. Moreover, assay sensitivity can be increased by increasing the sample volume up to the maximal incubation volume—i.e., 225 µL—or by increasing the antibody concentration (16). Further advantages of IFMA are the lack of radiation hazards and stability of the reagents. No detectable loss of sensitivity was observed over a period of three months.

The high sensitivity of the IFMA is partly a result of the time-resolved fluorometric assay technique and partly of the immunometric assay principle (16). When monoclonal antibodies become available, antibody consumption should present no problems, as indicated by recent developments in routine assays for human chorionic gonadotropin and lutropin (17, 18). The high sensitivity of the assay will facilitate studies of clinical conditions in which concentrations of

![Graph](image-url)
SmBP are subnormal, if they exist. It will also be useful for the characterization of SmBP in tissue extracts and cultures, where the content of SmBP can be minute.

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References