Enzyme Immunoassay for Erythrocyte Aldose Reductase

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This two-site immunoassay measures erythrocyte aldose reductase by using monoclonal and polyclonal antibodies to recombinant human enzyme. Total incubation time is 2.5 h, and the limit of detection is <0.05 μg/L. Analytical recovery tested with blood samples from healthy and diabetic individuals was 101–106%. Average CVs within and between assays were 3.7% and 4.8%, respectively. The enzyme content determined by this system correlated well with the activity of aldose reductase isolated from the same erythrocyte preparations. The amount of erythrocyte aldose reductase per milligram of hemoglobin was higher in women than in men (P < 0.001), but no significant correlation was observed between the amount of enzyme and the age of the individuals. This assay method should provide useful clinical information to optimize administration of aldose reductase inhibitors for effective prevention and treatment of diabetic complications.

Indexing Terms: diabetes/sex-related differences/enzyme inhibitors

The involvement of aldose reductase [EC 1.1.1.21; alditol:NAD(P)+ 1-oxidoreductase] in the pathogenesis of various diabetic complications has been implicated for more than two decades (1–7), and numerous aldose reductase inhibitors have been developed as possible therapeutic agents for diabetic patients. In some countries, including Italy and Japan, some aldose reductase inhibitors are already in clinical use to treat diabetic neuropathy (8). Aldose reductase is a member of the aldo-keto reductase family; members of this family coexist in most tissues. Using NADPH as a cofactor, aldose reductase catalyzes the reduction of various aldehydes, such as the aldehyde form of glucose, to the corresponding sugar alcohol, sorbitol.

Variable expression of aldose reductase among individuals is suggested by the inconsistent recovery of the enzyme from human placenta (9) and the different amounts of enzyme activity isolated from erythrocytes of diabetic patients (10). Because of the possible role of the sorbitol pathway in the development of diabetic complications, monitoring the concentrations of tissue aldose reductase as well as blood glucose may be essential for assessing the risk of developing complications involving this pathway. Data on the amount of aldose reductase in diabetic patients should also help clinicians optimize the administration of aldose reductase inhibitors when exploring the efficacy of these agents for clinical intervention against diabetic complications.

Until recently, no direct method was available to measure the amount of aldose reductase itself in human tissues. The estimation of enzyme content was mainly based on determining the activity of the enzyme when isolated from human tissues. Also present in most tissues, however, is alddehyde reductase [alcohol dehydrogenase (NADP+); EC 1.1.1.2], the substrate specificity of which overlaps with that of aldose reductase. This has necessitated laborious fractionation of these enzymes to determine how much of the activity is specific to aldose reductase. Previously, we reported (11) a two-site immunoassay developed with a polyclonal antibody to aldose reductase (ARAb) labeled with alkaline phosphatase, which we used to directly measure the amount of aldose reductase in specimens from human subjects. For a more practical application of this immunoassay to a larger number of samples, we report here the development of a convenient immunoassay for erythrocyte specimens, in which we use the Fab' fragment of a monoclonal antibody (mAb) generated against recombinant human aldose reductase (12, 13).

Materials and Methods

Procedures

Generation of monoclonal antibodies. Following the Guide for the Care and Use of Laboratory Animals, we immunized BALB/c mice intraperitoneally with 50 μg of purified recombinant enzyme mixed with complete Freund's adjuvant. We gave the mice a booster injection 3 weeks later. Three days after the boost, spleen cells of the mice were fused with SP2/0 myeloma cells, and antibodies secreted in hybridoma media were screened with immunoplates (Maxisorp F96; Nunc, Kamstrup, Denmark) coated with the antigen as previously described (14, 15). The isotype of the cloned antibodies was determined with a mouse mAb isotyping kit (Amer sham, Bucks, UK). For large-scale preparation, mAbs produced in mouse ascites fluid were precipitated with saturated ammonium sulfate and dialyzed against 0.1 mol/L triethanolamine (pH 7.7). The dialyzed sample was applied to a 2.6 × 30 cm Q Sepharose FF column

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6 Nonstandard abbreviations: ARAb, anti-aldose reductase polyclonal antibody; mAb, monoclonal antibody; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; ASAb, anti-aldose reductase monoclonal antibody 25; and HRP, horseradish peroxidase.
(Pharmacia Biotech, Uppsala, Sweden) equilibrated with 0.1 mol/L triethanolamine. The IgG fraction, eluted with a salt gradient (0–2.5 mol/L NaCl) in the same buffer, was further concentrated by ammonium sulfate precipitation and then dialyzed against phosphate-buffered saline (PBS; 150 mmol/mL NaCl, 1.9 mmol/L Na₂HPO₄, 8.4 mmol/L Na₂HPO₄, pH 7.4).

Immunoblot analysis and immunoprecipitation. To characterize the reactivity of isolated antibodies against the antigen, we incubated each mAb (10 mg/L) with a membrane on which 100 ng of purified recombinant aldose reductase was electroblotted after sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (16, 17). The bound mAb on the membrane was detected with 1000-fold-diluted peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dakopatts, Glostrup, Denmark) as described previously (11).

For immunoprecipitation, mAb25 (to aldose reductase) coupled to CNBr-activated Sepharose 4B (Pharmacia Biotech) was incubated with the soluble fraction of human sciatic nerve (11). The bound proteins and mAb dissociated from the Sepharose beads were separated on a 12.5% SDS–polyacrylamide gel and stained with silver (18).

Preparation of Fab' fragment and labeling with horseradish peroxidase (HRP). Purified mAb25 (2 g/L in PBS, 20 mL) adjusted to pH 4.0 with 1 mol/L citrate buffer (pH 3.2) was digested with 250 mg/L pepsin (Sigma Chemical Co., St. Louis, MO) at 37°C for 4 h. The reaction was terminated by adding 1/50 volume of 2 mol/L Tris-HCl (pH 9.0), and the fragments were fractionated on Superose 12HR16/50 (Pharmacia Biotech). Pooled Fab' fragment was concentrated with Centriprep 20 (Kurabo, Osaka, Japan) and dialyzed against 0.1 mol/L sodium phosphate buffer containing 5 mol/L EDTA (pH 6.0). 1.7 mL (4 g/L) of this Fab' fragment preparation we added 2-mercaptoethanol (final concentration 8 g/L) and then incubated the mixture at 37°C for 3 h. The reaction mixture was then loaded on Superose 12HR16/50 to obtain the reduced Fab' fragment.

The Fab' fragment was subsequently labeled with HRP (Toyobo, Tokyo, Japan), with use of N-(4-carboxycyclohexylmethyl)maleimide-N-hydroxysuccinimide ester (Zieben Chemicals, Tokyo, Japan) as described elsewhere (19). The Fab' fragment conjugated with maleimide-activated HRP was fractionated through Superose 12HR16/50 and stored at 4°C with 1 g/L bovine serum albumin (Sigma).

For preliminary characterization of the mAbs we directly labeled the purified antibodies with HRP, using sodium periodate as described (20).

The protein concentration was determined by the method of Bradford, calibrated with bovine gamma globulin (21). For the interference study, we used human hemoglobin from Cappel (Durham, NC), purified bilirubin and triglyceride from Sigma, and recombinant human insulin from Wako Pure Chemicals (Osaka, Japan).

Specimen Preparation

The following studies were performed in accordance with the principles of the Helsinki Declaration of 1975, as revised in 1983. After informed consent was obtained, blood from healthy adults was collected into heparin-containing tubes and transferred to tubes containing 0.15 mL of ACD-A solution (38 mmol/L citric acid, 85 mmol/L sodium citrate, 122 mmol/L dextrose) per milliliter of blood. Within 7 days of storage at 4°C, the erythrocyte fraction separated from plasma was washed and stored at -80°C as described previously (11). To prepare the hemolysate, we mixed the thawed erythrocyte fraction with an equal volume of 20 mmol/L phosphate buffer (pH 7.0). The erythrocyte suspension was subjected to two cycles of freezing and thawing in a solid CO₂–acetone bath. After centrifugation (15 000g, 15 min), the supernatant fraction of the hemolysate was stored at -80°C until the day of assay. The hemoglobin concentration of the lysate was determined by the method of Matsubara and Mimura (22).

ELISA of Aldose Reductase Mass

Immunoplates were coated at 4°C overnight with purified ARAb diluted in PBS (2 mg/L), 150 μL/well; all subsequent procedures were carried out at room temperature. Plates were washed five times with washing solution (0.5 mL/L Tween 20 in PBS), 300 μL/well, and blocked with blocking buffer (2 g of bovine serum albumin and 0.5 mL of Tween 20 per liter of PBS), 300 μL/well, for 1 h. We placed in duplicate wells, 100 μL of the antigen solution diluted in blocking buffer, sealed the wells, and incubated for 90 min (first incubation). After five washes with washing solution, we added to each well 100 μL of the labeled Fab' fragment in blocking buffer (150 μg/L), rinsed the wells, and incubated for 30 min (second incubation). After another five washes of the plate, we added 100 μL of substrate solution (0.5 g/L o-phenylenediamine and 75 mg/L hydrogen peroxide in purified water) to each well and incubated the samples for another 30 min in the dark. The reaction was stopped by adding 100 μL of 1 mol/L sulfuric acid to each well, and the antigen–Fab' complex formed was detected by measuring absorbance at 490 nm with a plate reader (Multiiskan Bichromatic; Labsystems, Helsinki, Finland).

Comparison Assay of Aldose Reductase Activity

Aldose reductase was isolated from erythrocytes by a modification of the method previously described (10). The entire procedure was performed at 0–4°C. About 10 mL of heparinized whole blood obtained from each individual was centrifuged at 1000g for 10 min. After removal of the buffy coat, the erythrocytes were suspended in 2 volumes of PBS and centrifuged again. This washing step was repeated once, and the packed erythrocytes were stored at -70°C until use.

Using a Model W200P Sonifer cell disruptor (Heat Systems-Ultrasonics, Plainview, NY), we homogenized 1.5 mL of the thawed erythrocytes with an equal volume
of 25 mmol/L imidazole-HCl buffer (pH 7.0) containing 5 mmol/L 2-mercaptoethanol. After centrifugation of the homogenate (20 000g, 15 min) we saved 40 µL of the supernate for hemoglobin determination. The rest of the supernate was desalted by passage through a DG10 gel-filtration column (Bio-Rad, Richmond, CA) equilibrated with the same imidazole buffer. The eluted protein fraction was then applied to a 0.7 × 18 cm PBE 94 polybuffer exchange column (Pharmacia Biotech), and the column was washed with 20 volumes of the imidazole buffer. The proteins were eluted with Polybuffer 74 (eightfold diluted, pH 4.0; Pharmacia Biotech) at a flow rate of 6 mL/h. The enzyme activity in each 2.5-mL fraction was measured as previously described (10). The activity of the first peak, separated from the second peak (derived from aldehyde reductase activity), was considered as the total activity of aldose reductase in the sample.

Results
Characterization of Monoclonal Antibodies

In all, 18 mAbs to aldose reductase were isolated and characterized. Two were of the IgM isotype; the rest were IgG1. To classify mAbs recognizing similar epitopes, we performed competition assays, using plates coated with antigen. Five mAbs were labeled with HRP, and the reaction of each of these was tested in the presence of each of the other unlabeled mAbs. The mAbs were consequently divided into two groups on the basis of possible overlapping epitopes. When cross-reactivity of the mAbs to aldehyde reductase was tested with plates coated with aldehyde reductase purified from human kidney (23), only one mAb (mAb25) demonstrated weak cross-reaction to the enzyme, which reportedly has partial amino acid sequence identity to aldose reductase (24).

Among the mAbs tested, mAb25 showed the strongest reactivity to the antigen—not only that immobilized on the plate but also in the free form—as detected by a two-site immunoassay with plates coated with mAb25. The specificity of mAb25 was verified by immunoprecipitation of tissue aldose reductase with mAb25-coupled Sepharose (Fig. 1). In the immunoblot analysis, however, mAb25 did not react to aldose reductase (data not shown).

Imunoassay Development

In preliminary experiments the highest sensitivity was obtained when mAb25 was used as the detector antibody and ARAb as the capture antibody in a two-step incubation. The times for the first and second incubations were set at 90 and 30 min, respectively, times when the first reaction was near completion and the second was >80% complete. For optimal color development we stopped the enzyme reaction at 30 min, although the reaction did not reach completion until after 2 h of incubation.

 Constructed with data for purified recombinant aldose reductase, the calibration curve was linear for concentrations of the enzyme ranging from 0 to 16 µg/L (Fig. 2). The lower limit of detection (mean + 2SD for the zero calibrator) was 0.034 µg/L.

Assay Characterization

Reproducibility. To determine within-run reproducibility, we analyzed erythrocyte samples from five diabetic patients in replicates of 10. The CVs varied from 2.5% to 4.3% (mean 3.7%). For between-run reproducibility, samples from five diabetic patients and nine healthy individuals were analyzed in 16 to 17 independent assays; the CVs were 3.1–6.5% (mean 4.8%). When the stability of aldose reductase in the hemolysate was examined after repeated freezing (−80°C) and thawing (25°C), no apparent change in enzyme concentration was observed among the aliquots of the same sample subjected to up to 10 cycles of freezing and thawing (data not shown).
Analytical recovery. Known amounts (1–8 μg/L) of purified recombinant aldose reductase were added to hemolysate samples from eight healthy and five diabetic individuals. The amount of total enzyme was measured and the recovery of the added aldose reductase was calculated after subtracting the amounts of endogenous enzyme in each sample. The mean percentage recovery in the samples of the two groups ranged from 101% to 106%.

Interferences. To samples containing 0 or 4 μg/L aldose reductase we added various endogenous substances—hemoglobin (≤2 g/L of final concentration), bilirubin (0.5 mg/L), triglyceride (4 mg/L), glucose (50 mg/L), NADPH (1 μmol/L), and insulin (2.5 mIU/L); hypoglycemic drugs—gliclazide (50 mg/L) and glibenclamide (2.5 mg/L); and aldose reductase inhibitors (1 μmol/L): epalrestat ([E,E]-5(2-methyl-3-phenyl-2-propenylidene)-4-oxo-2-thioxo-3-thiazolidine)acetic acid; Ono, Osaka, Japan), imirestat (2,7-difluoro-9H-fluorene-9-spiro-5′-'imidazolidine-2′,4′-dione; Alcon, Fort Worth, TX), methosorbinil (2-methyl sorbinil; Eisai, Tokyo, Japan), sorbinil (9)-6-fluorospiro(chrom-4,5′-imidazolidine)-2′,4′-dione; Pfizer, Groton, CT), stenal (3-[(4-bromo-2-fluorophenyl)methyl]-3,4-dihydro-4-oxo-1-phthalazineacetic acid; ICI, Cheshire, UK), and tolrestat (N-[6-methoxy-5(trifluoromethyl)-1-naphthalenyl]thioxomethyl]-N-methylglycine; Ayerst, Princeton, NJ). The effects of these substances were expressed as the percentage of absorbance (at 490 nm) for 4 μg/L of aldose reductase alone (corrected for the blank at 0 μg/L). No significant interferences were observed from the compounds tested.

Comparison with other methods. The amount of erythrocyte aldose reductase determined by immunoassay was compared with the activity of the enzyme preparation isolated from the same blood samples obtained from healthy and diabetic individuals. Although entirely different methods were used we observed a linear relation between the amount of enzyme protein and the activity of the isolated enzyme (Fig. 3).

The amount of erythrocyte aldose reductase determined in this study is about twice that reported in the previous study (11). This is not unexpected because in the current work we measured the concentration of the standard antigen solution (purified recombinant aldose reductase) by using bovine gamma globulin as the protein assay calibrator instead of the bovine serum albumin we used formerly. When we adjusted for the antigen concentration of the calibrator, the absolute values of erythrocyte aldose reductase (μg/g hemoglobin) measured by the current assay (y) were similar to those measured by the former assay (x): r = 0.981; y = 0.91x + 0.58 (n = 40, data not shown).

Clinical Values

As shown in Fig. 4, the amount of erythrocyte enzyme in 136 healthy individuals ranged from 6.3 to 16.9 μg/g hemoglobin. Men and women demonstrated statistically significant differences (P <0.001) in mean enzyme content. In 91 men the mean ± SD aldose reductase was 9.5 ± 1.6 μg/g hemoglobin; it was 11.3 ± 1.9 in 35 women.

![Fig. 3. Correlation between erythrocyte aldose reductase (AR) determined by the two-site immunoassay and by the activity assay of the isolated enzyme fraction.](image-url)

Hb, hemoglobin; □, samples from healthy individuals; ○, samples from diabetic patients. The regression equation for these 18 blood samples was y = 1.28x + 2.88 (SXY = 1.95, r = 0.88).

![Fig. 4. Amount of erythrocyte aldose reductase (AR) in healthy individuals.](image-url)

Each value (△, man; ○, woman) represents the mean of at least two separate assays.

No significant correlation was observed between the enzyme amount and the age of the individuals in each gender group.

Discussion

Compared with the method reported previously (11), this method not only yielded higher sensitivity and precision but also took markedly less total incubation time. Accordingly, this immunoassay system is suited for measuring the enzyme content in many blood samples at a time and should be useful in laboratories where aldose reductase inhibitors are already in clinical use as therapeutic agents for diabetic neuropathy or are being evaluated for such use (6). Our previous investigation of erythrocyte aldose reductase indicated a greater than twofold variability in the amount of this enzyme among healthy young adults (11). It is thus feasible to assume that the efficacy of aldose reductase inhibitors may be
associated with the variable amounts of aldose reductase in tissues of diabetic patients.

To further validate this possibility in a larger number of blood samples from diabetic patients, we improved the immunoblot by using the Fab' fragment of mAb25 labeled with HRP. This mAb demonstrated the strongest reactivity to the antigen in the immunosorbent assay and did not cross-react with the related enzyme, aldehyde reductase. However, the reaction of mAb25 to aldose reductase was conformation dependent. In the immunoblot analysis, mAb25 failed to react to aldose reductase, indicating that the epitope detected by mAb25 is susceptible to denaturation of the enzyme protein. Accordingly, the reactivity of mAb25 to aldose reductase may be lost when the enzyme changes its conformation during improper storage. Although we did not test the stability of the enzyme in hemolysate after prolonged storage at -80°C, the amount of enzyme remained steady after repeated freezing and thawing. This stability may be due to the exceptionally high concentration of protein, mainly hemoglobin, in the hemolysate preparation.

We observed no interference with the assay from various endogenous substances, hypoglycemic drugs, and aldose reductase inhibitors. In the present method we prepare blood samples by separating the erythrocyte fraction from plasma with two washing cycles with PBS before lysis. Consequently, the actual concentration of potentially interfering substances in the hemolysate should be much lower than the range we tested here.

Good correlation was obtained between the amount of aldose reductase protein determined by the immunoblot and the activity of the enzyme fraction isolated from the same erythrocyte samples. The determination of aldose reductase activity in erythrocytes requires careful handling of the samples to maintain constant recovery of the enzyme fraction as well as to avoid denaturing the enzyme protein during the isolation procedure. However, with the present immunoblot method we can avoid the step of isolating aldose reductase from the other enzymes in the aldo-keto reductase family.

The amount of erythrocyte aldose reductase per amount of hemoglobin was significantly higher in women than in men. Given the distinct gender difference in blood hemoglobin concentration, the higher value of erythrocyte aldose reductase demonstrated may be at least partly due to the lower amount of hemoglobin in the hemolysate obtained from women. Another possibility could be that the expression of aldose reductase in erythrocytes is influenced by hormonal environment. A recent study on 34.5-kDa mouse vas deferens protein, which is highly homologous to human aldose reductase, reported induction of the mRNA for this protein by testosterone (25). In rat ovary, on the other hand, the aldose reductase content fluctuated by more than twofold during the estrus cycle (N. Iwata, Tokyo Medical College, personal communication). Thus, the expression of aldose reductase in some tissues may be under the control of sex hormones. Certainly much remains to be clarified about this phenomenon.

As reported elsewhere (26), our investigation of diabetic patients indicates that the amount of erythrocyte aldose reductase determined by the previous immunoblot (11) is associated with the presence of retinopathy. Because that was a case-control study with a small number of blood samples, the finding must be further validated with a larger number of diabetic individuals. To elucidate whether the high erythrocyte aldose reductase is simply a result of retinopathy or indeed its causal factor, it will be necessary to monitor the enzyme content in patients during the course of the disease.

In conclusion, the high sensitivity, precision, and short incubation time of this improved immunoblot will aid rapid analysis of many blood samples from patients and should thus allow prospective studies to be performed on diabetic patients before they develop complications. Furthermore, the current method should provide useful data for optimizing administration of aldose reductase inhibitors when evaluating their efficacy in clinical intervention of diabetic complications.

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References