Antibodies to Porcine Uteroferrin Used in Measurement of Human Tartrate-Resistant Acid Phosphatase

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Materials and Methods

Materials

Uteroferrin: Pure porcine uteroferrin (8) was a generous gift from Dr. Philip Aisen, Albert Einstein College of Medicine, Bronx, NY.

Acid phosphatase preparations: Tartrate-resistant (type 5) acid phosphatase was partly purified from spleen from a patient with Gaucher’s disease, essentially as was described for purification from hairy-cell leukemic spleen by Ketcham et al. (7). However, after the gel-filtration through Sephadex G200 (Pharmacia, Uppsala, Sweden), we used affinity chromatography on a column of concanavalin A-Sepharose 4B (volume about 5 mL) instead of immunoaffinity chromatography, eluting with 30 mL of α-methyl-d-mannoside (500 mmol/L) in acetate buffer (10 mmol/L, pH 6.5) containing, per liter, 350 mmol of NaCl, 1 mmol each of MgCl₂, MnCl₂, and CaCl₂, and 300 g of ethylene glycol.

The final volume (31 mL) obtained from 500 g of spleen contained 1.1 mg of protein and 95 U of acid phosphatase activity per liter (specific activity, 85 kU per gram of protein). Only 5% of the activity was inhibited by d-tartrate. This specific activity is slightly less than that achieved by Ketcham et al. (7) at the same stage (approx. 110 kU/g, allowing for the different methods of assay), but we also experienced considerable losses during purification: our overall recovery was <10%.

Human lung and prostate tissue, obtained at autopsy, and placenta were extracted as previously described (2). A solution of erythrocytic acid phosphatase was obtained by lysing erythrocytes in water. Because of the lack of availability of suitable samples of human bone, we used sera from patients with increased tartrate-resistant acid phosphatase activity (due to Paget’s disease of bone) as sources of the osteoclast isoenzyme (“bone-disease serum”).

Procedures

Measuring enzyme activity: We measured acid phosphatase activity by an automated, continuous-monitoring assay based on the Hillmann procedure, with 1-naphthyl phosphate as substrate at pH 5.6, 37 °C (9), with or without addition of d-tartrate (40 mmol/L). In some experiments, e.g., when attempting to determine the amount of antibody-bound enzyme, we used a fixed incubation with 4-nitrophenyl phosphate. All activities are expressed as micromoles of substrate hydrolyzed per minute (U).

Immunization schedule: New Zealand White rabbits (3 kg) were immunized by combined intradermal, subcutaneous, and intramuscular injection of proteins in aqueous solution emulsified with equal volumes of Freund’s adjuvant. Uteroferrin antisera was obtained by inoculation with 1 mg of protein in Freund’s complete adjuvant, followed by another 1 mg similarly four weeks later. A further inoculation of 0.4 mg of uteroferrin in incomplete adjuvant was made after another six weeks, and serum was obtained two weeks later.
Purification of antibody: Antiserum for immunoprecipitation experiments was heated at 56°C for 30 min and clarified by centrifugation before use. Nonimmune rabbit serum was treated identically before use as a carrier.

Specific anti-uteroferrin antibody was purified from serum by affinity chromatography on columns containing uteroferrin-Sepharose 4B. Porcine uteroferrin (5 mg) was covalently coupled to 2.5 mL of swollen CNBr-activated agarose gel (Sepharose CNBr-CL4B, Pharmacia) at >90% efficiency, as recommended by Pharmacia. We diluted 100 mL of de-complemented antiserum with an equal volume of phosphate-buffered saline (PBS; NaCl 9 g/L, pH 7.4) containing 5 mmol of EDTA per liter; we then added 5000 kallikrein-inhibitory units of aprotinin (Bayer). The antiserum was twice passed through a column of uteroferrin-Sepharose (2.5 mL of gel), being eluted at a flow rate of 20 mL/h with 20 mL of PBS, followed by 10 mL of diluted PBS (equal volumes of PBS and water). Purified antibody was eluted from the column by addition of 10 mL of 20 g/L acetic acid solution. After neutralization with Tris base, the antibody was recovered by dialysis, then precipitated with saturated (NH₄)₂SO₄ at 4°C and centrifuged. We dissolved the pellet of antibody in the minimum volume of water and filtered this through a Sephadex G25 column (PD 10; Pharmacia) equilibrated with a solution containing 500 mmol of NaCl and 100 mmol of NaHCO₃ per liter, pH 8.6. Assuming the absorbance of a 10 g/L solution of rabbit IgG in a 1-cm light path to be 14, we calculated the yield of purified antibody by the procedure as approximately 5 mg.

Other procedures. We prepared anti-uteroferrin-Sepharose by coupling the antibody to CNBr-activated Sepharose CL 4B, as described above for the antigen-coupled gel. The concentration of protein was approximately 2 mg per milliliter of swollen gel.

Two-dimensional Ouchterlony immunodiffusion (10), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (11), and Western blot analysis (12) were carried out by standard techniques.

Results

Preparation and Characterization of Antiserum

Acid phosphatase purified from Gaucher's disease spleen proved to be only weakly antigenic when injected into the rabbit, presumably because of the low absolute amount of antigen protein in each injection. At most, only 20% of acid phosphatase added to serum was precipitated by this antiserum. With porcine uteroferrin as the immunogen, on the other hand, the antiserum produced by six weeks after the start of immunization could precipitate 90% of added acid phosphatase (Figure 1).

Titration curves for increasing amounts of spleen acid phosphatase added to human serum, as determined with two fixed volumes (20 or 40 µL) of antiserum obtained two weeks after the final inoculation, indicated that 1 µL of the antiserum was capable of precipitating about 1.25 mU of enzyme (Figure 2).

The rabbit anti-uteroferrin serum was further characterized by Western blot analysis. An extract of Gaucher's disease spleen, uteroferrin, and partly purified acid phosphatase separated in sodium dodecyl sulfate-containing gels were probed with antiserum. After washing the gels, we used ¹²⁵I-labeled Protein A to detect the immunoreactive species. In addition to the strong radioactive bands formed between the antisera and uteroferrin, bands of correspond-
### Table 1: M.W. KDa, UF, ACP, Spleen

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Fig. 3. Western immunoblots of partly purified acid phosphatase from Gaucher’s disease spleen (ACP; two center lanes, at different concentrations), the unfractionated spleen extract, and pure porcine uteroferrin (UF)

The blot has been overexposed with respect to uteroferrin to detect possible cross-reacting bands in the unfractionated spleen extract. The immunoreactive species were located with anti-uteroferrin antiserum, followed by 125I-labeled protein A. The anodal direction is downwards.

disease and in lung extract were too low to give well-defined precipitin arcs. However, Sepharose-bound anti-uteroferrin antibodies (see below) quantitatively precipitated tartrate-resistant acid phosphatase from bone-disease serum, and also from an extract of human lung. The respective acid phosphatase activities (total, tartrate-resistant, and immunoprecipitated) were: bone-disease serum, 6.5, 5.3, and 5.3 U/L; lung extract, 13.8, 12.0, and 11.8 U/L. Under the same conditions no activity was precipitated from an extract of prostate (acid phosphatase 1202 U/L) or erythrocytes (acid phosphatase 37 U/L, with 4-nitrophenyl phosphate substrate).

Use of Antiserum in Assays of Tartrate-Resistant Acid Phosphatase

The availability of an antiserum reacting specifically with human tartrate-resistant acid phosphatase allowed different designs of immunoassays to be tested: e.g., assays in which antibody-reactive activity is measured directly in the precipitate or bound to a solid phase, or assays in which the activity in the supernate is measured before and after reaction with antibody. An immunoassay in which the specific isoenzyme is separated by precipitation with antiserum and its activity determined in the precipitate allows the concentration of the analyte to be increased and so would be expected to offer greater sensitivity and precision than one in which the specific isoenzyme is determined from the difference between the total activity and the activity in the supernate after immunoprecipitation. Therefore, we first tested two immunoprecipitation assays, one with whole antiserum and one with Sepharose-bound purified antibodies.

We examined the correlation between added spleen acid phosphatase activity and the activity accounted for by resuspending the immunoprecipitate obtained with rabbit anti-uteroferrin antiserum. However, we could account for no more than 33%, less at low added activities (Figure 5). That these low recoveries were not due solely to incomplete precipitation of immunocomplexes was demonstrated by failure to increase them to above 50% by adding second precipitants such as anti-rabbit IgG or Polyethylene Glycol 8000. Antibodies to uteroferrin immobilized on Sepharose 4B have been used as an affinity reagent in the purification of spleen acid phosphatase (7). We therefore explored the use of purified antibodies bound to this matrix in an immunoprecipitation assay. After precipitation and centrifugation, bound acid phosphatase activity was eluted from the matrix with glycine–HCl buffer, 50 mmol/L, pH 2.3, containing 150 mmol of NaCl per liter (7). However, as reported previously (7), recoveries of bound acid phosphatase activity were low. Therefore, we did not explore this type of assay further and directed our attention to the assay of activities in solution after addition of antibody and in the supernate after precipitation.

Sepharose-bound anti-uteroferrin antibodies were suspended in four volumes of Tris buffer (10 mmol/L, pH 8.2, containing NaCl, 300 mmol/L). We added 0.3-mL portions of the suspension to 0.4 mL of the same buffer in glass centrifuge tubes with conical bottoms, then added 0.3 mL of a serum pool to which purified spleen acid phosphatase had been added to give various concentrations of activities. After mixing, we let the samples stand at 4 °C for 2 h, with occasional shaking. We then centrifuged them (1000 × g, 10 min) and quantified the acid phosphatase activity in 0.5 mL of each supernate by using 1-naphthyl phosphate as substrate, at 37 °C. The isoenzyme activity before precipitation was measured by diluting each sample to the same total volume, but without adding the antibody suspension. To measure nonspecific precipitation, we prepared controls to

Fig. 4. Ouchterlony immunodiffusion, showing (a), a reaction of immunological identity between anti-uteroferrin serum (center well) and the acid phosphatase activity of Gaucher’s disease spleen extract (well 1) and porcine uteroferrin (well 6), and no detectable reaction in wells 3 and 4, (prostatic extract) or wells 2 and 5 (erythrocyte extract); (b) reaction with spleen extract (wells 1 and 5) is again seen but no reaction with prostatic extract (wells 2 and 3), placental extract (well 4), or erythrocyte extract (well 6).

The gels were stained after washing with 1 g/L solutions of 1-naphthyl phosphate (a) or 2-naphthyl phosphate (b) and Fast Garnet GBC (1 g/L) in citrate buffer (100 mmol/L, pH 5.0). 2-Naphthyl phosphate was used in b to ensure detection of any precipitin arcs from erythrocyte acid phosphatase, which has low reactivity with the 1-naphthyl isomer.
Activities (U) added and precipitated are expressed per liter of sample in each case

Fig. 6. Acid phosphatase activity precipitated from serum by Sepharose-bound purified anti-uteroferrin antibodies, as a function of the quantity of added partly purified tartrate-resistant acid phosphatase from Gaucher's disease spleen

Activities (U) added and precipitated are expressed per liter of sample in each case

Discussion

Our results confirm the immunological similarity between porcine uteroferrin and the tartrate-resistant acid phosphatase isoenzyme of human spleen, previously reported for the enzyme from leukemic reticuloendotheliosis spleen (7) and reported here for the isoenzyme from Gaucher's disease spleen. These data provide additional evidence that the isoenzyme from these sources is identical with the tartrate-resistant acid phosphatase of osteoclasts and alveolar macrophages.

Anti-uteroferrin antibodies may offer advantages in designing an immunoassay for the tartrate-resistant isoenzyme in serum, in view of the difficulty of obtaining adequate amounts of this unstable isoenzyme for immunization. Of the possible assay configurations that we have tried, the assay by difference, with use of Sepharose-bound antibodies, was the most promising, but other possibilities such as the use of surface-immobilized antibodies may offer advantages if the low analytical recovery of antibody-bound activity can be overcome. The assay described by Lam et al. (6) measures activity in the precipitate after precipitation with an antiserum raised against the isoenzyme from hairy-cell leukemic spleen: however, only 42% of the activity was recoverable in the precipitate.

We have measured acid phosphatase activity in the assay by the automated modified Hillmann procedure used in our laboratory, to gain the advantages of a continuous monitoring method. The sensitivity of the immunoassay incorporating this method seems adequate at the upper reference limit for the tartrate-resistant acid phosphatase of serum from adults (about 5 U/L), as estimated for the same method of activity measurement and with addition of d-tartrate to improve isoenzyme specificity (9). However, 1-naphthyl phosphate is less rapidly hydrolyzed by the tartrate-resistant isoenzyme than is 4-nitrophenyl phosphate, and the linear molar absorptivity of the reaction product in the Hillmann procedure (15.6 · 10^3 L · mol^-1 · cm^-1) is rather less than that of 4-nitrophenol (18.6 · 10^3 L · mol^-1 · cm^-1). Therefore, sensitivity could be increased, presumably, by using the latter substrate in a fixed-incubation procedure. The amount of Sepharose-bound antibody used is adequate to bind all tartrate-resistant acid phosphatase in serum up to five times the upper reference limit, at least. The highest activity of this isoenzyme that we have observed was 21 U/L in the serum of a patient with osteoporosis; 1-naphthyl phosphate was the substrate.

In conclusion, our results demonstrate that cross-reacting antibodies raised against an antigen of nonhuman origin can form the basis of a practical and specific assay for human tartrate-resistant acid phosphatase. The assay offers a number of possibilities for further development.

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References


