Improved Electroimmunoassay of Factor VIII-Related Antigen

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Factor VIII-related antigen migrates poorly into gel during electrophoresis in agarose, probably in major part because of the relatively high sulfate content of the commonly used agars. A recently available low-sulfate residue agar, substituted for standard agar preparations in electroimmunoassay, allows Factor VIII antigen to travel more rapidly, producing “rockets” that are well defined and easily measured, making the assay easier and more reliable.

In the past several years interest has grown steadily in measuring Factor VIII-related antigen, particularly for use in hemophilia carrier diagnosis (1). The techniques currently in use for that purpose, radioimmunoassay and electroimmunoassay, appear to be of comparable reliability (2-5). Radioimmunoassay requires meticulous preparation of the radiolabeled antibody (no commercial product is available) and precipitation of Factor VIII with a carefully adjusted ammonium sulfate concentration (4). Electroimmunoassay, the “rocket” technique, (7), although less demanding in reagent preparation, calls for individualized adjustment of antibody concentration and electrical settings. An additional problem with the rocket technique results from poor migration of Factor VIII antigen in sulfate-containing gels, the net result being small rockets, which often are difficult to measure. A low electroendosmotic agar, low in sulfate content, has recently been made available, and this paper describes its application to Factor VIII testing.

Materials and Methods

Normal plasma pool: Blood was drawn from 12 men and 12 nonpregnant women by two-syringe technique. Nine parts of blood were added to one part of a solution of anhydrous sodium citrate (38 g/liter). The blood was put on ice and centrifuged (10,000 rpm, 15 min, 4 °C). Equal amounts of plasma were pooled, and 1-ml volumes were pipetted into polypropylene tubes. All but one tube were frozen at -80 °C. Serial dilutions of the nonfrozen sample were assayed for both Factor VIII activity and antigen and compared with the previous pool. A new pool was drawn at the end of each six-week period. For a new pool to be accepted for use, its antigen and activity values must vary no more than 5% from the previous pool.

Individual samples: Blood was drawn as in the protocol for the normal pool. After centrifugation the plasma of each sample was pipetted into four polypropylene tubes, three of which were immediately frozen at -80 °C and the fourth assayed both for Factor VIII activity and antigen. Samples for antigen were tested in duplicate on straight plasma and on a two-fold dilution. If Factor VIII activity was very high, antigen was assayed in duplicate in two- and fourfold dilutions. Samples were diluted with the barbital buffer (below) for antigen and in Owen’s buffer (sodium diethylbarbiturate, pH 7.35; Dade, Miami, Fla. 33152) for activity.

Antiserum: Human Factor VIII-associated protein antiserum was obtained from two sources: rabbit antiserum from Behring Diagnostics, Somerville, N.J. 08876 and goat antiserum from Atlantic Antibodies, Westbrook, Maine.

Buffer: Diethylbarbituric acid—sodium diethylbarbiturate (15 and 75 mmol/liter), pH 8.6, or Electrophoretic Buffer No. 2 (Fisher Scientific Co., Pittsburgh, Pa. 15219).

Electroimmunoassay: SeaKem HGT(P) Agarose (Marine Colloids, Rockland, Maine) was added to buffer, 10 g/liter, as was 350 mg of anhydrous disodium ethylenediaminetetraacetate. The solution was twice brought to boiling to ensure complete dissolution of agarose. A refluxing tube was used to minimize water loss. The agarose solution was then adjusted to 56 °C, and prewarmed (about 37 °C) antiserum was added (Behring, 36 ml of a fourfold dilution or Atlantic Antibodies, 5.0 ml of full-strength antiserum per liter). These figures varied with the lot. The mixture was poured by use of a prewarmed pipette into a prewarmed “sandwich” composed of a glass plate (10 × 20.5 cm), agarose-coated Mylar polyester film (3M Co., Minneapolis, Minn.) (11 × 20.5 cm, 0.1 cm thick plastic border), and glass plate (10 × 20.5 cm). The sandwich was held together by paper bulldog clips, three along the bottom side and one on each of the narrow sides. The Mylar was applied agarose side up to a wet glass plate. The exposed Mylar lip helped prevent poured agarose from leaking into the Mylar/glass side.

After solidification (about 30 min), the outer plate and border were removed. Seventeen wells, 3 mm in diameter and 1 cm apart, were cut in the agarose 2.5–3.0 cm from the long edge and were then aligned with the anodic side of the electrophoresis apparatus. Two sets of double wicks were cut from absorbent sheets (Gelman Instrument Co., Ann Arbor, Mich. 48106), 1.7 × 20 cm and 8.5 × 20 cm. The 1.7 × 20 cm wicks were placed in direct contact with the agarose, and the 8.5 × 20 cm wicks then completed the connection between the smaller wick and buffer chambers. Samples (1.5 µl) were applied by automatic pipette and electrophoresed for 18 h at 10 V/cm, then the resulting rocket was covered with filter paper, three layers of paper towels, and a glass plate. Pressure, 4.5 kg, was applied to the top of the plate for 10 min. The Mylar/agarose was washed for 30 min in saline (NaCl, 8.5
Results

A typical Factor VIII electroimmunoassay plate is shown in Figure 1. Low-sulfate agar has been used, and the gel has been mounted on a Mylar surface as described in the Methods section. Mylar, because it is thin and flexible, is convenient for storage. The undiluted pool sample, representing 100% antigen concentration, produces a rocket measuring about 34 mm, and serial dilutions down to 6.25% antigen concentration can be measured reproducibly without magnification. Figure 2 shows a standard curve constructed from these measurements; the shape does not change appreciably from run to run. Tested in quadruplicate in this system, a sample should not produce measurements that vary by more than 10% under standard operating conditions.

Discussion

Successful testing of potential hemophilia carriers requires that laboratories first assay Factor VIII activity and antigen as accurately as possible. Then, both measurements must be carried out simultaneously on fresh samples from a normal population of women and the results compared for reliability with published figures. Every laboratory step, including phlebotomy, must be standardized, and samples collected outside of the testing facility should be considered unacceptable (8). Once these procedures have been completed, testing of obligate hemophilia carriers and unknowns may be carried out reliably. The use of low-sulfate agar in Factor VIII antigen electroimmunoassay gives rise to rockets with higher amplitude, sharply defined, which are easily measured and translated into a standard curve that has a steeper slope, which allows for greater accuracy within the usual testing range. This technique, although still requiring considerable laboratory skill, should be easier to do successfully than electroimmunoassay with use of the usual gels. We hope that the technique modification reported here will contribute to the reliability of differentiating between carrier and normal females.

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References