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Biosensor for total antinuclear antibody determination at the point-of-care

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ABSTRACT

Antinuclear antibodies (ANA) are important in diagnosis and follow-up of patients with autoimmune conditions. The current increase in ANA requests is driven by broadening the use of ANA from a test for lupus to a test for diverse autoimmune diseases, but the standard method is protracted, cumbersome and prone to error. We describe an electrochemical method for quantifying total ANA for use as a point-of-care diagnostic aid. In this technology the target autoantigens are derived from a protein/nucleoprotein mixture prepared from an inexpensive source and adsorbed to a porous membrane with high protein binding capacity. Serum is slowly drawn through the membrane comprising the high density autoantigen mixture to induce rapid binding of patient autoantibodies. After rinsing, peroxidase-conjugated anti-IgG is drawn through the membrane followed by rinsing, insertion of an electrode assembly, and addition of the enzyme substrate. Substrate peroxidation is measured by microamperage-level current accompanying electrochemical reduction of the intermediate product. Values are comparable to a standard ANA test but require a total processing time of ~20 min. This method has the promise to greatly expand ANA testing in clinical settings for initial patient assessment of autoimmune disease.

1. Introduction

Detection of ANA in blood is widely used as an initial test to support the diagnosis of an autoimmune connective tissue disease (Agmon-Levin et al., 2014; Meroni and Schur, 2010; Wiik, 2005) although it is sometimes positive in infectious, neurologic, liver, or neoplastic disease (Damoiseaux et al., 2015). However, the standard ANA test requires specialized laboratory equipment and expertise and is generally performed in a licensed clinical laboratory, which must absorb the substantial set-up and operational costs. The requirement for transport to the testing lab, blood processing, test execution, and communication of results creates a cumbersome, time-consuming, error prone and expensive process that detract from its diagnostic value.

Point-of-care (POC) testing has the potential to overcome these problems and is showing increasing use in emergency settings and the operating theater (Price, 2001). Biosensor technology offers the promise to streamline laboratory testing, thereby improving productivity of health care systems by minimizing costs, time, and errors. If available, reliable POC testing for ANA could improve diagnostics in primary, urgent/emergency, and remote care clinics and enhance medical intervention for patients (Konstantinov et al., 2013).

We describe an electrochemical (EC) biosensor for quantitation of ANA with sensitivity and specificity comparable to that of the gold standard ANA test (Agmon-Levin et al., 2014) performed in the clinical laboratory. This device for measuring total ANA in fresh blood has a simple, economical, and flexible design, uses inexpensive disposable materials and reagents, and produces a relatively rapid readout suitable for routine patient assessment for underlying autoimmune disease.

2. Materials and methods

2.1. Human serum samples

Blood was obtained from donors under auspices of a human subjects protocol approved by the Institutional Review Board. After clotting, serum was removed by centrifugation and stored at 5 °C in the presence of 0.05% sodium azide for < 6 weeks prior to use. Most of the patients were diagnosed at the University of New Mexico Health Sciences Center Division of Rheumatology with a systemic autoimmune rheumatic disease based on accepted criteria. ANA titer was determined by indirect immunofluorescence

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by TriCore Reference Laboratories, Albuquerque, NM, USA using 2-fold serial dilutions beginning at 1:40 to a maximum of 1:2560. Donors of normal blood were anonymous employees of the university. All samples were assigned number codes and used in the biosensor without knowledge of clinical laboratory information.

2.2. Autoantigens preparation

Rabbit thymus acetone powder (RTP-1000, Immunovision) was suspended in cold phosphate buffered saline (PBS, 0.14 M NaCl, 0.01 M Na phosphate, pH 7.4 with 0.01% thimerosol) at a ratio of 2 g per 30 ml (67 mg/ml), and 5 mg phenylmethylsulfonyl fluoride (Sigma-Aldrich) was added. The mixture was stirred at moderate rate overnight at 5 °C. After centrifugation at 3000 RPM for 10 min, the supernatant was re-centrifuged at 10,000 RPM for 60 min This slightly turbid rabbit thymus extract (RTE) had a protein content of ~4.0 mg/ml (BCA assay, Pierce Biotechnology), and was stored in 1.0 ml aliquots at −70 °C. Chromatin (chr), which is the natural DNA-histone macromolecular complex present in the nucleus, was purified from calf thymus (Pel-Freez) and stripped of histone H1 as previously described (Burlingame and Rubin, 1990) to maintain solubility. The concentration of its DNA component was determined by absorbance at 260 mμ based on E=25 for 1 mg/ml, and protein content was determined by the BCA assay. Protein/DNA ratio of the chromatin preparations was ~1.27. It was stored in liquid form in 50% glycerol at −20 °C at a concentration of ~0.3 mg DNA/ml.

2.3. Autoantigen-coated membrane for immunosensor

Polyvinylidene fluoride (PVDF, Pall Life Sciences) 1.0 cm × 8.25 cm membrane strips were hydrated as previously described (Rubin et al., 2014). In the standard coating protocol, membrane strips were submerged in 8.0 ml of antigen solution consisting of 4.2 mg RTE+0.17 mg chromatin ("RTE+chr") diluted in PBS (RTE/chromatin ratio=25). After continuous tube inversion for 5 min, the membrane was stored overnight at 5 °C, rinsed with PBS, and post-coated with 0.1% bovine serum albumin (BSA, Sigma)+1.0% polyoxyethylene sorbitan monolaurate (Tween-20, stabilized and peroxide/aldehyde-free, Sigma) in PBS for 2 h at room temperature. Antigen-coated membranes were either used the same day or stored at 5 °C in PBS containing 0.005% NP-40 (Sigma-Aldrich) 'wash solution'.

For protein titration studies hydrated duplicate 0.75 cm squares of PVDF membranes were placed in 0.55 ml RTE+chr solutions of increasing concentration and processed as described above except for omission of the BSA component of the post-coating solution. Final wash solution was replaced with 0.1 ml PBS and membrane-bound protein determined by the BCA assay. For comparison to antigenicity, increasing RTE+chr concentrations were incubated with PVDF strips, processed as usual, and used in the immunoassay described below.

2.4. Amperometric immunoassay

A manifold machined into 8 independently accessible 0.8 cm diameter chambers exposing 0.5 cm^2 of the underlying membrane was used (Rubin et al., 2014). All steps are performed at room temperature. Two-tenths ml of human sera (diluted 1:50 in 'serum diluent' containing 2.5 mg/ml bovine gamma globulin (Calbiochem EMD Biosciences)+3.5 mg/ml BSA in wash solution) were added to each chamber and simultaneously drawn through the antigen-coated membrane over the course of 2.5 min at 0.08 ml/min using a weak vacuum. Each well was then sequentially rinsed with 0.1 ml and then 0.4 ml wash solution at 0.7 ml/min. Then 0.2 ml peroxidase-conjugated rabbit anti-human IgG (Southern Biotech) diluted ~1:10,000 in serum diluent was drawn through the membrane at 0.2 ml/min followed by 3 rinses with 0.1 ml, 0.4 ml and 0.4 ml wash solution at 0.7 ml/min. After manually inserting the electrode array (Alderon Biosciences), comprising 8 sets of screen-printed electrodes each consisting of a carbon graphite working electrode surrounded by a counter electrode and a silver chloride reference electrode, 0.15 ml peroxidase substrate solution consisting of commercially-stabilized hydrogen peroxide (H_2O_2)+2 mM 3,3′,5,5′ tetramethylbenzidine ('TMB liquid substrate system', Sigma) was added to each well. Product accumulation over time was measured by intermittent pulse amperometry in which TMB charge-transfer complex (Josephy et al., 1982) undergoes electrochemical reduction at the working electrode (set at −100 mV vs. the reference electrode) (Rubin et al., 2014). An amperometric reader (Alderon Biosciences) recorded current between the working and counter electrode at 2 min intervals using 20, 5 ms voltage pulses during 4 s with results reported as the average of the last 15 pulses as previously described (Rubin et al., 2014). Thirteen min was the standard assay endpoint. Electrode uniformity was evaluated using TMB charge-transfer product freshly-prepared by incubating TMB substrate for ~4 min in membrane coated with peroxidase anti-human IgG/human IgG until 10X diluted product reached ~2.0 OD at 370 mμ.

2.5. Composition analysis of preparations by Western blot (WB)

RTE and H1-stripped chromatin were heated in 'sample buffer' and separated at room temperature (RT) under reducing conditions by sodium dodecyl sulfate 15% polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (Konstantinov et al., 1996). For DFS-70/LEDGF analysis 62 μg RTE, 4 μg H1-stripped chr and 17 μg nuclear extract of HeLa cells (Active Motif) were subjected to SDS-PAGE. After electrotransfer to PVDF membrane for 1 h at 100 V and 4 °C, proteins were visualized by staining with Ponceau S. All subsequent steps were performed at RT, and blots were washed 3X for 10 min between steps with PBS containing 0.05% Tween-20 (PBST). Blots were blocked for 1 h with 5% non-fat dried milk in PBST and sequentially incubated for 1 h with a collection of reference human autoimmune sera diluted 100X (Konstantinov et al., 1996) followed by 2000X diluted horseradish peroxidase-conjugated rabbit anti-human IgG (Southern Biotech) in blocking buffer. Antibody specificities of prototype sera were confirmed by WB and by indirect immunofluorescence microscopy at 1:100 dilution using HEp2 cells (Inova Diagnostics) and visualized with an Olympus IX70 microscope and digital camera. Prestained protein molecular markers (Fisher) were used to calibrate the immunoblots. Bound antibodies were detected by chemiluminescence (Clarity substrate, BioRad) according to the manufacturer's instructions.

2.6. ANA detection by enzyme-linked immunosorbent assay (ELISA)

ELISA for autoantibody reactivity to chromatin and/or RTE was employed as previously described (Burlingame and Rubin, 1990) except that antigen coating of RTE+chr wells was done by a sequential addition protocol in which RTE at 20 μg/ml was added 3 h after incubating wells with chromatin at 0.8 μg/ml (RTE/chromatin ratio=25). Post-coating wells with RTE after chromatin had no effect on detection of anti-chromatin antibody (using anti-RTE-negative sera, n=6, data not shown) but resulted in an average 30% reduction in anti-RTE reactivity compared to RTE alone (using anti-chromatin-negative sera, n=5, data not shown) presumably due to chromatin-mediated loss of RTE adsorption sites on the polystyrene wells.