Glucose Oxidase Immunoenzyme Methodology as a Substitute for Fluorescence Microscopy in the Clinical Laboratory

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Enzymes as markers for antigens or antibodies in immunohistochemical procedures have several advantages over commonly used fluorochrome labels. These include use of a regular light microscope and the ability to get permanently stable slide preparations. Glucose oxidase (EC 1.1.3.4), being absent in mammalian tissue, provides no background staining, such as that seen with the commonly used horseradish peroxidase (EC 1.11.1.7) owing to peroxidase-like activity in tissues. A glucose oxidase histochemical method is detailed that is useful for detection of human antibodies; it can be easily used in clinical laboratories as a substitute for fluorescent techniques.

Additional Keyphrases: immunoglobulins • histologic staining • enzyme-antibody conjugates • anti-nuclear antibodies

Although enzyme immunohistochemical procedures have been available in the clinical laboratory for some time, preference has been given to methods involving fluorochrome labels. Most of the immunohistochemical studies have involved horseradish peroxidase conjugates (1–3), which present problems of background staining from endogenous peroxidase-like activity in mammalian tissues. Efforts to quench this endogenous activity can result in a change or loss of substrate antigenicity. Glucose oxidase (β-D-glucose:oxygen 1-oxidoreductase; EC 1.1.3.4), a microbial enzyme, is known to give no background in mammalian tissues and to react with available tetrazolium salts to create high-contrast and easily recognized morphologic patterns (4). Additionally, the tetrazolium salts used for glucose oxidase histochemical reactions are not known to be carcinogenic, unlike the high-contrast substrate, diaminobenzidine, used in horseradish peroxidase staining (5). The use of immunoenzymic methods has definite advantages. These procedures do not need an expensive fluorescent microscope, and produce slides that are permanently stable.

Glucose oxidase immunohistochemical procedures have been used to detect the presence of polypeptide hormones such as gastrin (6), viral antigens such as respiratory syncytial virus (6), and bacterial antigens such as Legionella pneumophila (7). We have successfully used this methodology for the detection of human immunoglobulins, e.g., anti-nuclear antibodies, and present some preliminary results.

Materials and Methods

Human serum samples. Patients' sera with anti-nuclear antibody titers ranging from <20 to ≥230 were used. Comparison fluorescent assays were performed with a commercial anti-nuclear antibody kit (Electro-Nucleonics, Bethesda, MD 20014). Within this group of samples, different sera demstrated by the fluorescence method each of the four anti-nuclear antibody reactivity patterns: homogeneous, speckled, rim, and nucleolar. For screening with the glucose oxidase immunoenzymic technique, each serum was diluted in serial two-fold dilutions as used with the fluorescence procedure for detection of anti-nuclear antibodies.

Antibody conjugation with glucose oxidase: Antibody-enzyme conjugates were prepared with a two-step glutaraldehyde cross-linking procedure, with a mixture of affinity-purified goat antibodies specific for human mu- and gamma-chains (Antibodies Inc., Davis, CA 95616) (6). For conjugate standardization we used a method similar to that of Walwicz et al. (8).

Disclosing reagent. The disclosing solution consisted of the enzyme substrate, α-D-glucose; an electron-transport agent, phenazine methosulfate; and a final electron receptor, a tetrazolium salt. Reduction of the tetrazolium salt results in the deposition of the corresponding formazan precipitate at the site of the original antigen–antibody reaction. Different tetrazolium salts have been used, including: 2,2'-di-p-nitrophenyl-5,5'-diphenyl-5,5'-dimethoxy-4,4'-diphenylene)ditetrazolium chloride (p-nitro blue tetrazolium chloride, p-NBT); 2,2',5,5'-tetra-p-nitrophenyl-3,3'(3,3'-dimethoxy-4,4'-diphenylene)ditetrazolium chloride (tetrabromo blue tetrazolium chloride, TNBT); and 2-(b2-benzothiazolyl)-5-styryl-3-(4'-phthalhydrazidyl)tetrazolium chloride (BSPT).3 These sera were obtained from Research Organics, Inc., Cleveland, OH 44125, and Sigma Chemical Co., St. Louis, MO 63178.

The disclosing reagent was prepared in NaH2PO4, 0.1 mol/L. pH 6.9. The final concentrations, per liter, were 41.7 mmol (7.5 g) of α-D-glucose and 326 μmol (0.1 g) of phenazine methosulfate. The concentration of tetrazolium salt varied: p-NBT was 2.45 mmol/L (0.5 g/L); TNBT and BSPT were 0.25 g/L—exact concentrations depended on the solubility of the salt. For convenient measurement and minimal waste of reagents, we prepared 20 mL of the disclosing reagent at a time. After agitation, the mixture was stored in the dark at 4 °C for at least 1 h before use. This allowed mutarotation of the α-glucose to the β-anomer. Before use, we filtered the mixture through a 0.22-μm (average pore size) filter to remove any undissolved salt and any contaminating bacteria and fungi.

Immunohistologic staining. We used two substrates for detection of anti-nuclear antibodies: cells from human KB line (Electro-Nucleonics) and rat kidney sections (obtained from Cleveland Clinic, Cleveland, OH). These materials were rehydrated in phosphate-buffered saline (0.01 mol/L NaH2PO4, 0.15 mol/L NaCl, pH 7.4) at room temperature. After rehydration, 25 μL of diluted human test serum was applied to the substrates. The slides were placed in a moist chamber at room temperature for 30 min, after which they were washed three times with phosphate-buffered saline; each

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3 Nonstandard abbreviations: p-NBT, p-nitro blue tetrazolium chloride (see text for comprehensive name); TNBT, tetra nitro blue tetrazolium chloride (see text); BSPT, 2-(b2-benzothiazolyl)-5-styryl-3-(4'-phthalhydrazidyl)tetrazolium chloride.
washed lasted 3 min. We then applied to the sections goat specific antibody (to human mu- and gamma-chain) conjugated to glucose oxidase. The sections were again washed three times with phosphate-buffered saline as before. The disclosing solution, containing one of the tetrazolium salts as previously described, was applied to the sections for 45 min at 37°C in a moist chamber. The sections were then washed and mounted in polyvinyl alcohol, if p-NBT or TNBT was used, or mounted in Permount (a naphthalene polymer in toluene), if BSPT was used. The characteristic pink formazan precipitate obtained when BSPT was used appeared to be leached from the substrate in aqueous media; therefore, these slides were dehydrated in ethanol and xylene and mounted in a nonaqueous media such as Permount. The blue-black precipitate from p-NBT and the black precipitate from TNBT appeared to be stable in mounting medium with aqueous or nonaqueous base. Because mounting with aqueous polyvinyl alcohol omits the dehydration step, it was the preferred method. After mounting the slides, we viewed them under a regular microscope. Staining patterns were assessed without prior knowledge of the fluorescent assay results.

Results and Discussion

Some of the serum samples that produced different anti-nuclear antibody staining patterns in the fluorescence procedure were tested with the glucose oxidase method on human KB line and rat kidney tissue sections. Figure 1 demonstrates that glucose oxidase immunoenzymic conjugates can be successfully used with BSPT to demonstrate homogeneous patterns of anti-nuclear antibody reactivity with human KB cells. Figure 2 shows nucleolar and peripheral or rim patterns, as clearly demonstrated with formazans from TNBT on human KB cells. Figure 3 demonstrates the homogeneous pattern of anti-nuclear antibody reactivity on rat kidney substrate. Figures 4 and 5 show the lack of background staining on KB cells and rat kidney sections, respectively, for serum with no detectable anti-nuclear activity.

We cannot directly compare the titers obtained with glucose oxidase conjugate and fluorescence conjugate because the two were prepared from different mixtures of anti-human antibodies. The evaluation of immunoenzymic techniques involving horseradish peroxidase label, compared with fluorescence methods, for the detection of anti-nuclear antibodies has been reported by Walwick et al. (8).

Glucose oxidase-conjugated antibody techniques offer two significant advantages over other immunolocalizing methods for use with a light microscope. First, unlike fluorochrome or peroxidase labels currently in use, glucose oxidase offers an entirely negative background, which greatly facilitates morphologic evaluation. Secondly, glucose oxidase can be used with numerous tetrazolium salts that are not known to be carcinogenic. The salts can be mounted in either aqueous polyvinyl alcohol for semipermanent preparation or with Permount for a permanent record. Glucose oxidase immunoenzymic localizing methods compare favorably with fluorescence methods except that a longer time is involved in the
staining reaction, because of the additional step involving application and incubation of the disclosing reagent.

The reagents necessary for the glucose oxidase procedure are stable for at least six months under optimum conditions, and the working solutions of antisera and conjugate are stable for at least one week. The disclosing reagent, however, can be used for only two or three days after being prepared. The phenazine methosulfate is light sensitive and also appears to cause spontaneous reduction of the tetrazolium salt upon prolonged storage.

Currently, the main drawback of the use of glucose oxidase-labeled antibody conjugates is their lack of commercial availability. The methodology can be easily used in most clinical laboratories that have a light microscope and the means to produce enzyme conjugates.

References


