

Glucose oxidase as label in histological immunoassays with enzyme-amplification in a two-step technique: coimmobilized horseradish peroxidase as secondary system enzyme for chromogen oxidation

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Summary

A sensitive staining procedure for glucose oxidase (GOD) as marker in immunohistology is described. The cytochemical procedure involves a two-step enzyme method in which GOD and horseradish peroxidase (HRP) are coimmobilized onto the same cellular sites by immunological bridging or by the principle of avidin-biotin interaction. In this coupled enzyme technique, H₂O₂ generated during GOD reaction is the substrate for HRP and is utilized for the oxidation of chromogens such as 3,3'-diaminobenzidine or 3-amino-9-ethylcarbazole.

Due to the immobilization of the capture enzyme HRP in close proximity to the marker enzyme (GOD), more intense and specific staining is produced than can be obtained with soluble HRP as coupling enzyme in the substrate medium. Indirect antibody labelled and antibody bridge techniques including the avidin (streptavidin)-biotin principle have proven the usefulness of this GOD labelling procedure for antigen localization in paraffin sections. Antigens such as IgA in tonsil, alpha-fetoprotein in liver and tissue polypeptide antigen in mammary gland served as models.

The immobilized two-step enzyme procedures have the same order of sensitivity and specificity as comparable immunoperoxidase methods. The coupled GOD-HRP principle can be superior to conventional immunoperoxidase labelling for the localization of biomolecules in tissue preparations rich in endogenous peroxidase activities.

Introduction

Besides peroxidase and alkaline phosphatase, the fungal enzyme glucose oxidase (β -D-glucose: oxygen 1-oxidoreductase, EC 1.1.3.4) can be employed as a marker enzyme in immunohistology. The activity of glucose oxidase (GOD) is usually visualized with tetrazolium salts and intermediate electron carriers (Nachlas et al. 1957; Reiss 1966; Clark et al. 1982), but grained cytochemical stainings with overall background due to settled formazan deposits as well as fading and diffusion of color are often obtained. Alternatively, the staining of GOD by a coupled peroxidase procedure and by use of an appropriate chromogen may be performed (Fig. 1). It was proposed for such a technique that the secondary system enzyme,

i.e. horseradish peroxidase (HRP) is simply added to the reaction mixture quite analogous to assays in clinical chemistry (Kuhlmann 1970; Kuhlmann and Avrameas 1971).

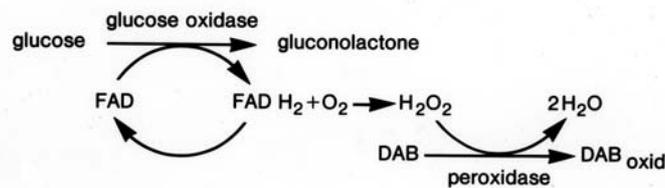


Fig.1. Schematics of glucose oxidase (GOD) staining by the two-step enzyme technique using horseradish peroxidase (HRP) as secondary system enzyme. Hydrogen peroxide formed in the oxidation of β -D-glucose by molecular oxygen is the substrate for HRP and further utilized for the oxidation of 3,3'-diaminobenzidine (DAB) to yield a colored product (DAB_{oxid})

In its original use, this enzyme-amplified principle often gives a drawback at the light microscopic level by generation of imprecise staining with diffuse localization of cellular details. On testing incubation conditions further, we found those unfavorable properties to be attributable to the soluble HRP in the substrate mixture. In the meantime, multistep enzyme systems have shown that a tightly coupled enzyme system will work better inasmuch as a cumulative effect on the efficiency of the overall reaction is achieved (Mosbach and Mattiasson 1976). Thus, we coimmobilize the secondary system amplifier (HRP) in close proximity of the marker enzyme (GOD) onto the same cellular sites by immunological or avidin-biotin bridges and, consequently, excellent localization of antigen is achieved.

The usefulness and the sensitivity of this new method is proven by immunolocalization of IgA in tonsil, alpha-fetoprotein in liver and tissue polypeptide antigen in mammary gland. For this purpose, indirect antibody labelled and antibody bridge techniques including the avidin-biotin method are employed.

Materials and methods

Immunological reagents and biochemicals. Primary rabbit immune sera against human IgA and rat alpha-1-fetoprotein (AFP) are prepared as described (Kuhlmann 1975; Kuhlmann and Peschke 1985). Rabbit antibodies against tissue polypeptide antigen (TPA) are purchased from Sangtec Medical (Bromma, Sweden).

Goat anti-rabbit IgG antibodies and rabbit anti-goat IgG antibodies are isolated from respective hyperimmune sera, conjugated with GOD or HRP and purified (Kuhlmann et al. 1974; Kuhlmann 1984). Soluble peroxidase-antiperoxidase antibody complexes (PAP) from rabbit and goat are prepared by the method of Sternberger et al. (Sternberger et al. 1970).

For the avidin-biotin system, goat anti-rabbit IgG antibodies, rabbit anti-goat IgG antibodies and GOD are biotinylated via biotinyl-N-hydroxysuccinimide (BNHS) to achieve an amino group substitution of about 70% for antibodies and an amino group substitution of 40-50% for GOD (Guesdon et al. 1979). Streptavidin (Calbiochem. GmbH, Frankfurt, FRG) is coupled to glutaraldehyde activated HRP (Kuhlmann et al. 1974). Reagents for the preparation of conventional avidin-biotin-peroxidase complexes (ABC) are obtained from Vector Laboratories (Burlingame, CA, USA).

GOD (250 U/mg) and HRP (isoenzyme C, RZ 3.2-3.3) are obtained from Boehringer-Mannheim (Mannheim, FRG), BNHS from Calbiochem. GmbH (Frankfurt, FRG); catalase (30,000-40,000 U/mg), 3-amino-9-ethylcarbazole (AEC) are from Sigma Chemicals (München, FRG) and pronase E from *Streptomyces griseus* (approx. 8 DMC-U/mg) from Serva-Labor (Heidelberg, FRG). All other chemicals are from Merck (Darmstadt, FRG).

Tissue specimens. Human tonsils and mammary glands are fixed in 8%-10% formaldehyde for 12-24 h at room temperature, then processed routinely and embedded in paraffin. Liver blocks from carcinogen treated and hepatoma bearing rats (Kuhlmann 1978) are fixed in ice-cold 99% ethanol-1% acetic acid for 12-18 h, dehydrated and embedded in paraffin; 5-7 μm thick sections are mounted on acetone cleaned and bovine serum albumin (BSA) conditioned slides (Kuhlmann 1978; Kuhlmann and Peschke 1984). Sections are deparaffinated in xylene and passed through ethanol into 0.01 M phosphate buffer pH 7.2 supplemented with 0.15 M NaCl (PBS). Formaldehyde fixed specimens are further treated with pronase (Denk et al. 1977; Kuhlmann and Peschke 1985).

Immunolocalization by immobilized two-step enzyme system. The incubation schedules of enzyme conjugated antibody methods as well as modified PAP and avidin-biotin bridge techniques are summarized in Table 1. Nonspecific binding is blocked by incubation of tissue sections with 20% normal serum (same species as that which provides the Sandwich antibodies); blocking of endogenous avidin binding sites are not necessary with the specimens employed here. Sera, antibodies, PAP and streptavidin-biotin-enzyme complexes are diluted in PBS supplemented with 1% BSA (PBS/BSA). Unreacted reagents are rinsed off with three successive washes for 5 min each in PBS/BSA.

Table 1. Immunolocalization of antigens with GOD as marker by the immobilized two-step enzyme system GOD-HRP

Incubation	Antibody conjugated method bridging GOD and HRP conjugated antibodies	Antibody conjugated method bridging GOD conjugated antibodies and PAP complexes	Avidin-biotin complex method bridging biotin-antibody and complex from avidin-HRP and biotin-GOD
Step 1	Primary antibodies from rabbit 10 $\mu\text{g}/\text{ml}$ ^a Control: nonimmune normal serum or primary antibody omitted	Primary antibodies from rabbit 1 $\mu\text{g}/\text{ml}$ Control: nonimmune normal serum or primary antibody omitted	Primary antibodies from rabbit 0.2 $\mu\text{g}/\text{ml}$ Control: nonimmune normal serum or primary antibody omitted
Step 2	GOD labelled goat anti-rabbit IgG working dilution 1:30 Control: unlabelled antibodies or GOD labelled rabbit anti-goat IgG	GOD labelled goat anti-rabbit IgG (linking antibody) 1:30 diluted Control: unlabelled antibodies or GOD labelled rabbit anti-goat IgG	Biotin labelled goat anti-rabbit IgG 10 $\mu\text{g}/\text{ml}$ conjugate Control: biotin labelled rabbit anti-goat IgG or unlabelled goat anti-rabbit IgG antibodies
Step 3	HRP labelled rabbit anti-goat IgG (HRP labelled goat anti-rabbit IgG antibodies work as well) working dilution 1:50 Control: this step omitted	Rabbit PAP complexes working dilution 1:50 Control: this step omitted or replaced by goat PAP complexes	Avidin-biotin complexed enzymes: 5.0-10 $\mu\text{g}/\text{ml}$ streptavidin-HRP plus 5.0 $\mu\text{g}/\text{ml}$ biotinylated GOD ^b Control: conventional avidin-biotin-peroxidase complex
Step 4	Glucose-DAB reaction	Glucose-DAB reaction	Glucose-DAB reaction

^a Concentrations and working dilutions are established by preliminary experiments. Incubation in primary antibodies for 18 h at 4° C, incubation in other reagents for 20-30 min at room temperature

^b Routinely employed mixture deduced from preliminary experiments with various ratios of biotinylated GOD and HRP conjugated streptavidin

In the case of avidin-biotin experiments, complexes are prepared 30 min prior to use by appropriate mixture of streptavidin-HRP conjugate and biotinylated GOD (see Table 1). Optimal concentrations are determined in preliminary experiments analogous to the formation of ABC complexes in the conventional avidin-biotin-peroxidase technique (Hsu et al. 1981).

For control purposes, primary antibodies, conjugates, PAP complexes and streptavidin-biotin complexed enzymes are replaced by unrelated reagents (Table 1); also, sections are simply incubated in step 3 and step 4 reagents.

In order to prove the efficiency of the new GOD staining method (the immobilized two-step enzyme system), antigens are also stained by a GOD technique in which the secondary system enzyme HRP is simply added to the substrate mixture (Kuhlmann 1970; Kuhlmann and Avrameas 1971). Furthermore, conventional immunoperoxidase methods are employed for comparison (Hsu et al. 1981; Kuhlmann 1984).

GOD histochemistry and cytochemical control reactions. Stock solutions of D(+)-glucose are prepared in advance to enable mutarotation to the β -isomer. The reaction medium contains 10 mg β -D-glucose and 0.5 mg DAB per ml 0.01 M phosphate buffer pH 6.8 (10 min saturated with O₂). Slides are incubated for 30 min at room temperature, washed, dehydrated and mounted in resinous medium under coverglass. Apart from DAB as chromogen, AEC (Graham et al. 1965) is employed as well.

Endogenous GOD activity is checked on tissue sections by reaction in glucose-DAB substrate without prior immunohistological incubations. Specificity is also verified on immunoreacted sections which are incubated in the following media: a) glucose omitted as substrate or replaced by galactose; b) addition of 0.02 M sodium pyruvate to the complete medium (Aebi et al. 1962; Venkatachalam and Fahimi 1969) for inhibition of liberation of H₂O₂; c) addition of 0.1% catalase to the complete substrate in order to intercept H₂O₂ which is generated by the action of GOD on glucose and thus abolishes cytochemical staining; d) addition of 0.05 M 3-amino-1,2,4-triazole to the substrate medium in order to inhibit endogenous catalase (Margoliash et al. 1960; Venkatachalam and Fahimi 1969), so preventing its potential oxidase and peroxidase activities.

Results

The immobilized two-step enzyme technique reveals GOD activity in either of the employed immunolocalization methods in an intense, reproducible and clear-cut manner. For example, the carcino-fetal antigen AFP is strongly stained in hepatocarcinoma cells while surrounding normal liver cells do not react (Fig. 2). The other studied antigens are also detected in their typical histological sites as shown for IgA in human tonsil (Fig. 3) and for TPA in mammary gland epithelia and carcinoma cells (Fig. 4). With soluble HRP as secondary system amplifier, i.e. HRP simply added to the glucose-DAB substrate mixture instead of being coimmobilized in the proximity of GOD, antigen localization is less precise (Fig. 5). Moreover, patchy and confluent staining is also observed.

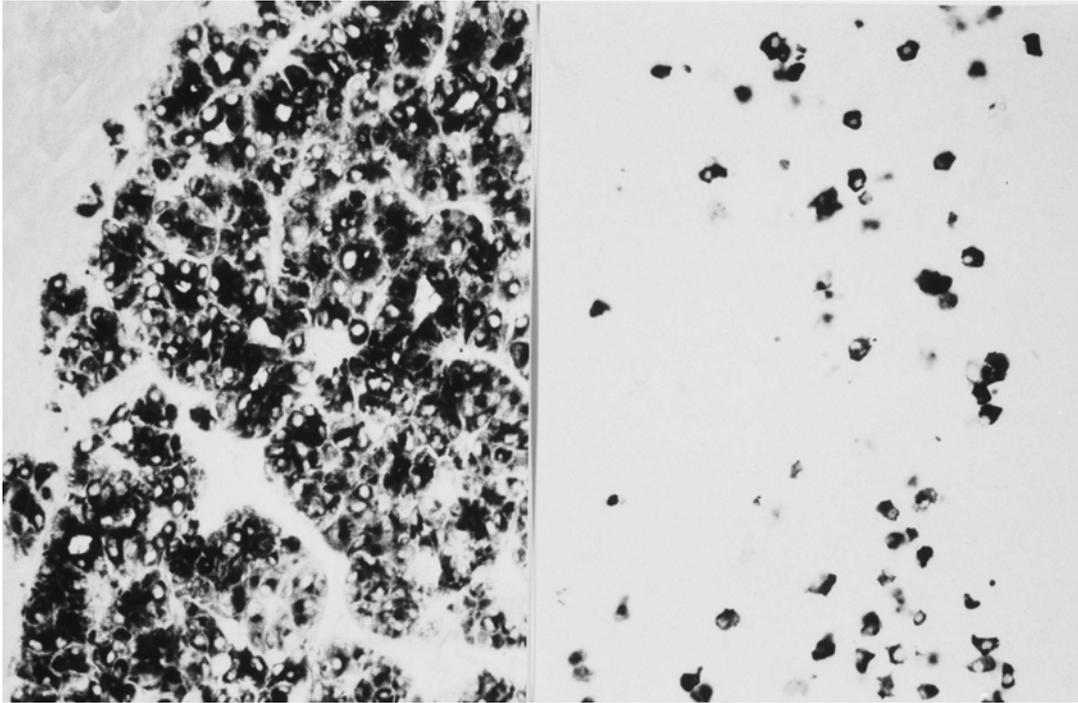


Fig. 2. Localization of AFP by the coimmobilized GOD-HRP principle in paraffin section from rat liver bearing a hepatocellular carcinoma. Indirect method with GOD and HRP labelled antibodies. Note clear and intense reaction in cytoplasm of hepatoma cells. Original x 160

Fig. 3. Paraffin section from formaldehyde fixed human tonsil stained for IgA by coupled GOD-HRP principle using streptavidin-biotin procedure. Strong IgA reactivity in cytoplasm of plasma cells in extrafollicular area. Original x 250

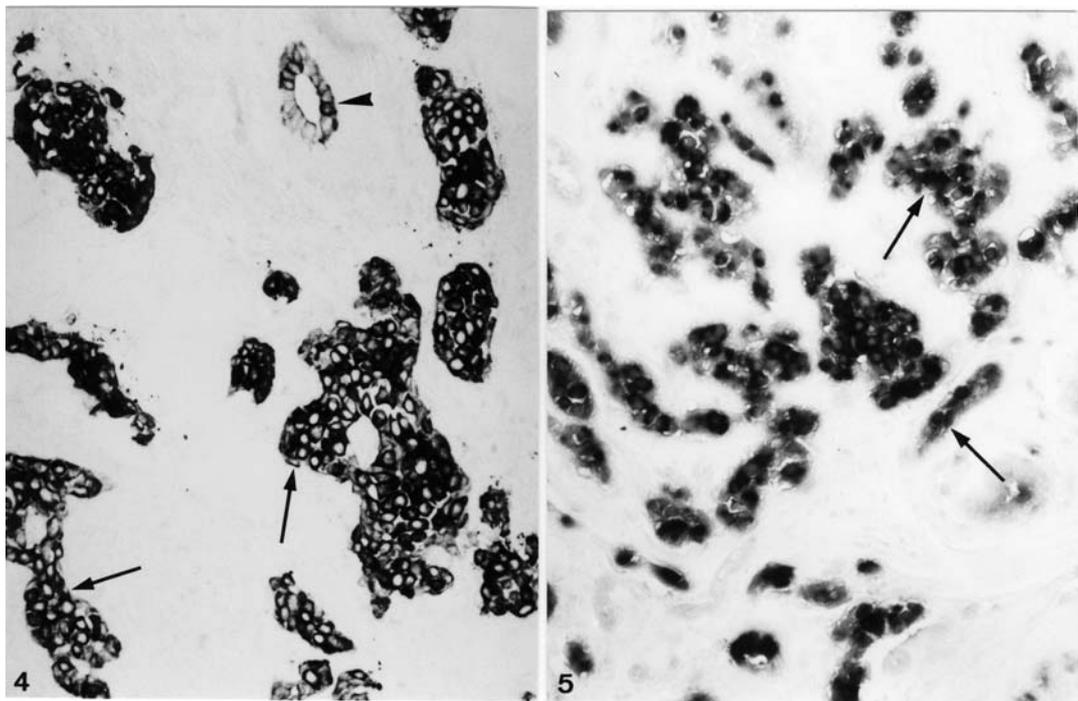


Fig. 4. Paraffin section from formaldehyde fixed human breast carcinoma stained for TPA by the new GOD-HRP technique. Rabbit PAP complexes bridged to GOD labelled second antibody (goat anti-rabbit IgG) and reacted with glucose-DAB substrate. Note clear positive staining in mammary carcinoma cells and in duct epithelium. Original x 160

Fig. 5. Section from same tissue block as Fig. 4 and stained for TPA by GOD-HRP principle; indirect method with GOD labelled sandwich antibody in which HRP as secondary system enzyme is simply added to the substrate medium. Cellular sites with TPA reactivity are confluent and less precise than in Fig. 4. Original x 160

Antigen localization and appearance of the enzyme reaction product are almost identical to those in serial sections reacted with conventional immunoperoxidase methods. This also holds true when DAB is replaced by AEC as chromogen. Experiments with primary antibody dilutions have shown that the here described labelling procedures have the same order of sensitivity and specificity as comparable immunoperoxidase methods.

No staining is observed when tissue sections without prior antibody incubations are reacted with complete substrate medium, and when in the incubation sequence unrelated immunoreagents, PAP complexes or avidin-biotin systems are employed so that either the marker enzyme (GOD) or the "helper" enzyme (HRP) is missing. Then, no staining is seen when glucose is omitted or replaced by galactose in the substrate medium and when sodium pyruvate or exogenous catalase are added to the complete medium. In the latter case, H_2O_2 generation by GOD action is prevented or H_2O_2 is intercepted so that DAB cannot be oxidized. Addition of aminotriazole to GOD substrate has no influence on cytochemical antigen staining.

Discussion

The coupled GOD-HRP principle was developed for the use of GOD as marker enzyme in immunohistology because no such endogenous enzyme activity is known in mammalian cells and especially in order to profit from the general usefulness of DAB as cytochemical stain. For this purpose, it became evident that only the coimmobilization of both enzymes, i.e. bridging of GOD and HRP to the same cellular site, proves to be a reliable tool. Thus, more intense and specific stains are generated than are obtained with soluble HRP as secondary system enzyme.

The new method is easily performed. The clarity and intensity of cytochemical reaction compares well to that obtained with immunoperoxidase procedures. The same holds true for the sensitivity, specificity and morphological appearance of the enzyme reaction product. Reagents can be readily prepared or commercially purchased. Moreover, and this is very important for the protection of antigenicity, we observe with our tissue specimens that the coimmobilized GOD-HRP staining principle does not require inhibition of endogenous peroxidases as is usually necessary for conventional immunoperoxidase procedures. Preliminary experiments show that the described method is also suitable for other immunoenzyme techniques such as ELISA and immunoblot methods.

Several control experiments demonstrate the specificity of the immunocytochemical reaction. The coupled GOD-HRP principle is specific for the detection of GOD activity. It requires the presence of specific substrate (glucose; O_2 saturation of buffer is not a prerequisite) and is suppressed by addition of catalase or sodium pyruvate (degradation of H_2O_2 and inhibition of H_2O_2 liberation) to the enzyme substrate. The absence of endogenous peroxidase activity demonstrates the efficiency of the HRP capture reaction which prevents diffusion of the GOD generated H_2O_2 .

Oxidation of DAB may occur in tissue sites under certain conditions in which endogenous peroxidases can act on H_2O_2 liberated by previous oxidation of endogenous substrates (Graham and Karnovsky 1965). In such cases, appropriate inhibitors are suggested. Also, specimen preparations in which electron accepting oxidases can oxidize DAB spontaneously,

thus interfering with specific antigen localization, will require inhibitory pretreatments. False positive and false negative results due to endogenous catalase are readily avoided by addition of aminotriazole to the substrate medium (Margoliash et al. 1960; Venkatachalam and Fahimi 1969). However, in our specimens, such an inhibition is not needed.

Generally, the avidin-biotin principle can involve problems of nonspecificity especially due to the use of egg white avidin. This may be overcome with high pH and strong molarity buffer (Hsu et al. 1981; Bussolati and Gugliotta 1983). We thus prefer streptavidin from *Streptomyces avidinii* because nonspecific protein-protein interactions are minimized in tissue sections at physiological pH due to its physical characteristics (Morris and Saelinger 1984).

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