GLUCOSE OXIDASE AS AN ANTIGEN MARKER FOR LIGHT AND ELECTRON MICROSCOPIC STUDIES

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Summary

A new staining procedure for glucose oxidase at both the light and the electron microscopic level has been developed. The procedure was equally effective for the detection of galactose oxidase. It appears that the method can be applied to all oxidoreductases which catalyze the production of hydrogen peroxide. *Aspergillus niger* glucose oxidase and *Dactylium dendro-ides* galactose oxidase were used to immunize rabbits and mice in order to trace the distribution of specific antibodies in the immunocytes of the popliteal lymph nodes.

Introduction

Complexes formed by enzymes and their homologous antibodies possess enough catalytic activity to allow the detection of antienzyme antibodies. On the basis of this observation, enzymes of various origins were used to study the synthesis of antienzyme antibodies in immunocytes (1, 4, 5, 18, 19). For the studies at the ultrastructural level mainly peroxidase (3, 11, 17) or alkaline phosphatases (13, 15) were used.

In the present study we have used another enzyme, glucose oxidase of *Aspergillus niger*, in order (a) to show that rabbits and mice can produce specific antiglucose oxidase antibodies, (b) to demonstrate that it is possible to obtain an electron-dense reaction product for this enzyme and thus to localize the antiglucose oxidase antibodies at the ultrastructural level and (c) to find out whether synthesis of antiglucose oxidase antibodies. Furthermore, galactose oxidase was also used in order to show that the staining reaction developed for the glucose oxidase can be utilized to detect any enzyme which upon action on its substrate produces hydrogen peroxide.

MATERIALS AND METHODS

Materials: Glucose oxidase of *A. niger* (110 IUB units/mg) and galactose oxidase of *Dactylium dendroides* (25 units/mg) were obtained from Worthington Biochemical Company; glucose oxidase (140 units/mg) and horseradish peroxidase (RZ 3) were purchased from CF Boehringer Mannheim. Bovine serum albumin (Povite, Amsterdam) was also used.

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Histochemical staining for enzymes: At the light microscopic level glucose oxidase activity was detected following a published procedure utilizing β -D-glucose as the substrate and MTT tetrazolium salt as hydrogen acceptor (2). Galactose oxidase activity was demonstrated by the same procedure but substituting β -D-galactose for D-glucose.

Glucose oxidase activity was also revealed by a procedure analogous to that reported by Keston for the colorimetric measurement of D-glucose activity in biologic fluids (9). When the usually employed chromogenic hydrogen donors (*o*-toluidine, *o*-dianisidine, 2,6-dichlorophenolindophenol) were replaced by the diaminobenzidine reagent of Graham and Karnovsky (6), a colored, insoluble and osmiophilic material was obtained that revealed the presence of glucose oxidase activity at either the light or the electron microscopic level (Fig. 1).

The incubating medium contained 15 mg β -D-glucose, 0.5 mg diaminobenzidine tetrahydrochloride and 1 mg horseradish peroxidase (RZ 3) per ml 0.1 M phosphate buffer, pH 6.8. For light microscopy the incubation time was 10-15 min, while for electron microscopy it was 1 hr. In the latter case the incubating medium was renewed with a freshly prepared mixture after 30-min incubation. Galactose oxidase activity was demonstrated by the same procedure but substituting β -D-galactose for β -D-glucose.

Immunization procedure: One-year-old rabbits (giant Flemish) and 3-month-old C_{57} black mice were used for immunization. The animals received the antigen by subcutaneous injection in the hind footpads. Rabbits were injected with 5 mg glucose oxidase in 0.5 ml 0.15 M NaCl dispersed in 0.5 ml complete Freund's adjuvant (Difco Laboratories). Mice were injected with 0.25 mg galactose oxidase in 0.025 ml 0.15 M NaCl dispersed in 0.025 ml complete Freund's adjuvant. The animals were bled and killed 18-25 days after a single antigen injection, and the popliteal lymph nodes were excised. Control animals were immunized either with bovine serum albumin or with horseradish peroxidase.

Light microscopy: Cell suspensions from lymph nodes were prepared, smeared on slides and fixed as described previously (3). The slides containing the fixed cells were incubated for 30 min in buffered saline containing 0.1 mg/ml glucose oxidase or galactose oxidase. The slides were then washed with buffered saline and the sites of bound enzymes were revealed with one of the staining procedures described above.

Electron microscopy: Blocks, 1-2 mm³, of popliteal lymph nodes from rabbits or mice were fixed for 24 hr at 3°C in a 4% formaldehyde solution, freshly prepared from paraformaldehyde (Merck), in 0.2 M cacodylate buffer, pH 7.3 (10). The tissues were washed in several changes of cacodylate buffer for 24 hr at 3°C. The tissue blocks were then cut into extremely small fragments with a razor blade or frozen sections of 40 μ were prepared in a cryostat (Dittes-Duspiva). Incubation of small fragments or sections was carried out at room temperature for 24 hr in cacodylate buffer containing 3 mg/ml of either glucose oxidase or galactose oxidase. Other small fragments were incubated for 1 hr at room temperature in cacodylate buffer containing 10% dimethyl sulfoxide and 3 mg/ml enzyme antigen. The reaction mixture was then rapidly frozen with liquid nitrogen, slowly warmed to room temperature and finally allowed to stand for 23 hr at room temperature. After incubation, the tissues were washed for 20-30 min in three changes of buffer and were immediately treated with the appro-priate Substrate as described above.

All control tissues were prefixed as indicated. The specificities of the immunologic and cytochemical reactions were examined as follows: (*a*) lymph nodes stimulated by glucose oxidase or galactose oxidase were incubated in the substrate medium alone or with the corresponding enzymes and revealed with the substrate mixture from which glucose or galactose was omitted; (*b*) the same lymph nodes were incubated with horseradish peroxidase and peroxidase activity was revealed with 3,3'-diaminobenzidine (DAB) and H_2O_2 (6); (*c*) lymph nodes stimulated with unrelated antigens, i.e., horseradish peroxidase and or bovine serum albumin, were incubated with glucose oxidase or galactose oxidase oxidase and then stained with their corresponding substrates; further the peroxidase-stimulated lymph nodes were incubated for 5

min and 1 hr with peroxidase and subsequently stained with Graham and Karnovsky's medium, thus revealing synthesis of specific antihorseradish peroxidase antibodies in the immunocytes.

After postfixation in cacodylate-buffered 2% OsO_4 , the tissues were dehydrated in ascending series of ethanol and embedded in Epon (12). The small tissue fragments were polymerized in gelatin capsules and the 40 μ sections were flat embedded in the lids of BEEM capsules. Epon-embedded tissues were cut with a Porter Blum MT-1 ultratome and examined without any further staining or after treatment for 30 sec with lead citrate (14) using a Siemens Elmiskop I operated at 80 kV with a 50 μ objective aperture.



FIG. 1. Schematic representation of the staining procedure.

FIG. 2. Light micrograph of a 40 μ frozen section showing the medullary region of a popliteal lymph node of a rabbit 18 days after immunization with glucose oxidase. Plasma cells containing antibody to glucose oxidase are strongly colored (arrows). X 1000.

FIG. 3. Blast cell containing antiglucose oxidase antibody in the perinuclear space and a few ergastoplasmic cisternae. Small tissue fragment, 20 days after immunization. Section was not counterstained. X 15,600.

At the light microscopic level, the staining of glucose oxidase utilizing the coupled peroxidase procedure was found to be as specific and sensitive as that obtained by the MTT technique (Fig. 2). In addition, no diffusion of the staining occurred as was sometimes the case with the tetrazolium technique. Furthermore, the use of the peroxidase reaction as the final step permitted the detection of antiglucose oxidase antibodies in immunocytes with the electron microseope.

Antibodies were observed in a continuous sequence of immunocytes from the antibodyproducing blast stage to the final mature plasma cell. In the blast cells antibody was detected in the perinuclear space and in a few ergastoplasmic cisternae (Fig. 3). During the differentiation of the immunocyte, the antibodies were detected either in all of the ergastoplasmic cisternae (Fig. 5) or, most often, only in some of them (Fig. 4). Often antibodies were localized in the peripheral cisternae of the cell. In the latter case the perinuclear space might or might not contain antibodies (Fig. 6). In few immunocytes antibodies were present in the Golgi apparatus without staining of any of the ergastoplasmic cisternae (Fig. 7). Detection of galactose oxidase antibody gave similar results (Fig. 8).

Lymph nodes stimulated with glucose oxidase or galactose oxidase showed no reaction when incubated in the substrate medium alone or incubated with the enzymes followed by incubation in the histochemical medium lacking glucose or galactose. When the same lymph nodes were incubated with horseradish peroxidase and subsequently with DAB and hydrogen peroxide, endogenous peroxidase activity was detected in certain cell lines such as eosinophils and red blood cells (*cf.* references 3, 11 and 18). When tissues stimulated by the injection of horseradish peroxidase or bovine serum albumin were incubated with glucose oxidase or galactose oxidase and their corresponding substrates, no intracellular enzyme activity was revealed, but occasionally the contrast of the cell surface membrane was enhanced similarly to that seen in the experimental samples (Figs. 4 and 6). The incubation of peroxidase-stimulated lymph nodes with peroxidase for 1 hr followed by the reaction with DAB and hydrogen peroxide showed synthesis of specific antihorseradish peroxidase antibodies in immunocytes, thus demonstrating a secretory immune response (Fig. 9).

Peroxidase was shown to penetrate rapidly into the intracellular spaces. Thus peroxidasestimulated lymph nodes were incubated with the antigen for not longer than 5 min, washed and then stained. A positive reaction was observed in immunocompetent cells (Fig. 10).

DISCUSSION

The purpose of the present work was to find whether oxidoreductases could be used, in addition to peroxidase (3, 11, 17) and alkaline phosphatase (13, 15), as antigen markers at the electron microscopic level. Glucose oxidase and galactose oxidase were used because no such endogenous activities were observed in the immunocytes in lymph nodes and because these two enzymes are available in highly purified form from commercial sources. Glucose oxidase of *A. niger* is a glycoprotein containing 2 molecules of flavine adenine dinucleotide and has a molecular weight of 185,000 (20). Galactose oxidase of *D. dendroides* is a metalloenzyme containing 1 g copper/mole and has a molecular weight of 45,000 (8).

At the light microscopic level glucose oxidase has been utilized to detect antiglucose oxidase antibodies (1, 4, 5, 7), while glucose oxidase-labeled antibodies were used to reveal cellular antigens (1, 2). In these studies the enzyme was detected by utilizing the MTT tetrazolium technique.

In the present work with the coupled peroxidase technique glucose oxidase activity was detected at the light microscopic level by the yellowish brown color of oxidized DAB and at the electron microscopic level by its osmiophilic properties (6). The same technique was used

with success for the ultrastructural localization of galactose oxidase. It is then quite possible that the procedure can be applied for the detection of any other enzyme which upon action on its substrate produces hydrogen peroxide.



FIG.4. Plasma cell synthesizing antibody to glucose oxidase. Reaction product is present in the perinuclear space and in a part of the cisternae (arrow). Most of the rough endoplasmic reticulum is negative. Enhanced contrast of the plasma embrane can be seen at the upper right (arrowhead). Same tissue as in Figure 3; 40 μ frozen section. Lead citrate staining for 30 sec. X 15,000.

FIG. 5. Young plasma cell containing antiglucose oxidase antibody in the perinuclear space and in the cisternae of the rough surfaced endoplasmic reticulum, 18 days after the injection of glucose oxidase. Small tissue fragment. Lead citrate staining. X 15,000.



FIG. 6. Plasma cell synthesizing antibody to glucose oxidase. The perinuclear space and some peripheral cisternae are strongly stained (arrows). Note partially enhanced contrast of the cell surface membrane (arrowhead); 18 days after immunization. Small tissue fragment with one freeze-thaw cycle in the presence of 10% dimethyl sulfoxide. Section was not counterstained. x 13,000.

FIG. 7. Same tissue and procedures as shown in Figure 6. Note antiglucose oxidase antibody in the Golgi apparatus. X 15,600.

FIG. 8. Plasma cell containing antibody to galactose oxidase in the cisternae of the rough endoplasmic reticulum; 40 μ frozen section of a lymph node 18 days after a single injection of galactose oxidase. Section was counterstained with lead citrate. X 15,600.



FIG. 9. Part of a plasma cell containing antibody to horseradish peroxidase as revealed by incubation in peroxidase for 1 hour, followed by DAB anf H_2O_2 ; staining reaction in the lumina of the cisternae. Rabbit lymph node, 18 days after a single injection of peroxidase, 40 μ frozen section. Thin section was not counterstained. X 15,000.

FIG. 10. Same tissue as shown in Figure 9; incubation time for peroxidase antigen was not longer than 5 min; enzyme activity was revealed with DAB and H_2O_2 . Reaction product is present in the rough endoplasmic reticulum near the cisternal membranes; 40 μ frozen section. No counterstaining. X 28,000.

In our experiments antibody could be detected ultrastructurally when small blocks were used. However, by this procedure the exact position of the block as related to the lymph node structure was difficult to appreciate. Furthermore, penetration of the enzyme and the substrate reagent into the interior of the blocks was difficult to achieve, as discussed in another paper (10), while the freeze-thaw cycle seemed to improve penetration. The most satisfactory results were obtained when 40 μ frozen sections were used. Using this procedure, penetration of the enzyme was more regular and easier. In addition, the 40 μ sections once stained and embedded in Epon could be examined under the light microscope and the positive areas as related to the lymph node structure could be established.

The localization of specific antibody in our experiments was comparable to that of antiperoxidase and antialkaline phosphatase antibodies described by other authors (3, 11, 15, 17), thus supporting the specificity of the cytochemical reaction.

The advantage of using glucose oxidase as an antigen marker is based on its high specificity. In the lymph nodes examined no glucose oxidative activity was observed, as was the case with peroxidase or alkaline phosphatase.

In our controls nonspecific intracellular reactions were not observed, as was apparent from the different immunization schedules and the various incubation procedures utilized. However, cell surface membranes occasionally showed enhanced contrast after incubation with glucose oxidase and with the reagents employed to reveal the glucose oxidase activity. We have observed a similar phenomenon in the case of the peroxidase-antiperoxidase system, especially after glutaraldehyde fixation. This type of plasma membrane staining may be caused partly by aldehyde prefixation of tissues that produces active groups capable of crosslinkage. As additional controls, peroxidase-stimulated lymph nodes were incubated either with glucose oxidase or with peroxidase. No reaction was observed after incubation for glucose oxidase activity, whereas we could establish a secretory immune response for antiperoxidase antibodies. In order to demonstrate a rapid intracellular penetration of macromolecules we shortened the incubation time for the peroxidase antigen to 5 min. Even with such a short incubation time we could observe intracellular antigen-antibody complexes. Seligman *et al.* (16) utilized a reaction mixture containing catalase for the demonstration of mitochondrial enzyme activities. Since catalase has a 6-fold higher molecular weight than of horseradish peroxidase, it appears that with suitable precautions macromolecules can penetrate fixed tissues and can be utilized for electron microscopic procedures.

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