ENZYME-LABELED ANTIBODIES

FOR THE LIGHT AND ELECTRON MICROSCOPIC

LOCALIZATION OF TISSUE ANTIGENS

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ABSTRACT

Enzymes, either acid phosphatase or horseradish peroxidase, were conjugated to antibodies with bifunctional reagents. The conjugates, enzymatically and immunologically active, were employed in the immunohistochemical localization of tissue antigens utilizing the reaction product of the enzymatic reaction as the marker. Tissues reacted with acid phosphatase-labeled antibodies directed against basement membrane were stained for the enzyme with Gomori's method, and those reacted with peroxidase-labeled antibody were stained with Karnovsky's method. The reaction products of the enzymes localized in the basement membrane. Unlike the preparations of the fluorescent antibody technique, enzyme-labeled antibody preparations were permanent, could be observed with an ordinary microscope, and could be examined with the electron microscope. In the latter, specific localization of antibody occurred in the basement membrane and in the endoplasmic reticulum of cells known to synthesize basement membrane antigens. The method is sensitive because of the amplifying effect of the enzymatic activity. The ultrastructural preservation and localization were better with acid phosphatase-labeled antibody than with peroxidase-labeled antibody, but acid phosphatase conjugated antibody was unstable and difficult to prepare. Peroxidase-antibody conjugates were stable and could be stored for several months at 4°C, or indefiniely in a frozen state.

INTRODUCTION

The fluorescein-labeled antibody or immunofluorescent method developed by Coons and his co-workers (4) for the localization of tissue antigens is specific and sensitive and has been employed widely with the light microscope. Among the deficiencies of the technique, which are admittedly few in number, are a lack of permanence of the preparations which require a fluorescent microscope for their observation and a masking of the specific fluorescence by the naturally occurring fluorescence of tissues. The latter can be troublesome in tissues rich in elastic tissue, for instance. For the ultrastructural localization of tissue antigens, ferritin (26, 27) or heavy metals (19, 29, 30) have been used because fluorescein lacks electron opacity. These techniques for the ultrastructural localization of antigen, which offer so much to the cell biologist, are not being used widely because of inherent technical difficulties. Owing to the large molecular size of ferritin, ferritin-labeled antibody penetrates tissues poorly (22, 26) and heavy metal-labeled antibody has provided insufficient increase in contrast at the sites of antigen-antibody reactions to be useful (22, 26). Recently, better contrast has been obtained with heavy metals by a new but complex technique (29).

In search of a better label, histochemically

demonstrable enzymes of small molecular weight were conjugated to antibodies by employing bifunctional reagents (17, 23). The enzymatically and immunologically active conjugates were reacted with tissues which then were stained histochemically for the enzymes, with deposition of reaction products at the antigenic sites. In addition to obtaining permanent preparations for light microscopy, the electron opacity of the reaction products proved useful for the ultrastructural localization of antigens.

In order to demonstrate the simplicity and usefulness of the enzyme-labeled antibody method, the present paper reports a light and electron microscopic study of an epithelial basement membrane antigen (EBM) known to be synthesized by epithelial cells (20, 21).

MATERIALS

Acid phosphatase and horseradish peroxidase were used as the enzymatic labels, since they were obtainable from commercial firms, and were stainable histochemically at light (2, 18) and electron microscopic levels (6, 11), and since their endogenous localization was limited to certain well-defined sites (6, 18). Wheat germ acid phosphatase was obtained from Worthington Biochemical Corporation, Freehold, N.J., and horseradish peroxidase (Crude, type II and type VI) from Sigma Chemical Co., St. Louis, Mo.

The gamma globulin fraction of rabbit anti-mouse epithelial basement membrane (anti-EBM) or anti-mouse collagen (20) and the gamma globulin fraction of sheep anti-rabbit globulin each were conjugated to enzymes by using bifunctional reagents, either p,p'-difluoro-m,m'-dinitrodiphenyl sulfone (FNPS) (General Biochemicals, Div. North American Mogul Products Co., Chagrin Falls, O.) or 1-ethyl-3-(3-dimethylamino propyl) carbodiimide (CDI) (Ott Chemical Company, Muskegon, Mich.). For the immunohistochemical localization of antigen, the spleens, testes, and a parietal yolk sac carcinoma of strain 129 mice were employed. The parietal yolk sac carcinoma has been shown to synthesize large amounts of a basement membrane antigen which is localized in all epithelial basement membranes of the mouse but which fails to cross-react with connective tissue antigens (16, 21).

DEVELOPMENT OF METHODS

Preparation of Acid Phosphatase-Labeled Antibodies

Acid phosphatase was conjugated to antibodies by using FNPS (24) or CDI (7). Since the alkaline con-

ditions of the FNPS reaction tended to inactivate the enzyme, the reaction with CDI was preferred (23). For this, 200 mg of acid phosphatase, 200 mg of anti-EBM and 500 mg of CDI were dissolved in 6 ml of distilled water. The mixture was allowed to react for 30 min at room temperature with gentle agitation, dialyzed against 0.8% saline overnight, and centrifuged to remove denatured and precipitated protein.

In order to isolate the acid phosphatase–anti-EBM conjugate from the unreacted acid phosphatase and unreacted anti-EBM, the reaction mixture was fractionated on a column of Bio-Gel P-300 with saline as eluent. For each fraction, the protein content was determined spectrophotometrically at 280 mμ and the acid phosphatase activity by the method of Lowry et al. (13) which utilizes para-nitrophenyl phosphate as substrate. The presence of acid phosphatase–antibody conjugate in each fraction was determined by reacting the fraction with tissue sections with morphologically recognizable sites containing antigen and then subjecting the tissue sections to the histochemical reaction for acid phosphatase.

The bulk of the protein material was eluted at the void volume of the column (tube No. 16) and followed by a small peak at tube No. 24 when the reaction mixture was fractionated. When the unconjugated anti-EBM alone was fractionated through the column, some proteinous material was eluted also at the void volume and followed by a peak at tube No. 26. The unconjugated acid phosphatase was eluted at tube No. 30 and followed by a secondary peak at tubes No. 51 through 53 (Fig. 1).

The acid phosphatase activity of the reaction mixture fraction eluted from the void volume to tube No. 24 was greater than that of the unconjugated acid phosphatase fraction. This indicates that the acid
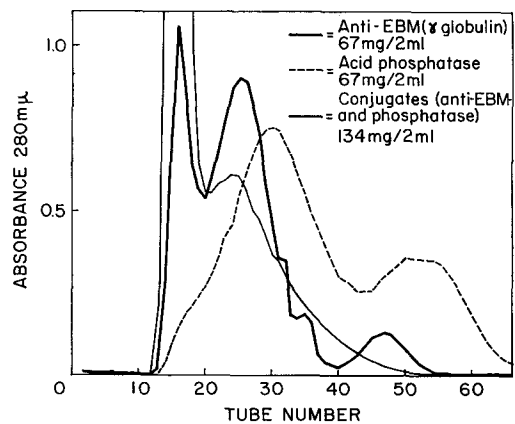

FIGURE 1 Fraction patterns of acid phosphatase-anti-EBM conjugate, unconjugated acid phosphatase and unconjugated anti-EBM. Column height: 28 cm. Volume per fraction: 3.0 ml.

phosphatase eluted in these tubes was of a larger molecular size than the unconjugated acid phosphatase which was eluted several tubes later (Fig. 2). The secondary peak of the unconjugated acid phosphatase fraction had no enzymatic activity. The fractions obtained from tubes No. 23 through 26 stained the epithelial basement membrane of the parietal yolk sac carcinoma; this indicates that these fractions contained the immunologically and enzymatically active conjugate.

Acid Phosphatase-Labeled Antibodies for Localization of Tissue Antigen

Slices of parietal yolk sac carcinoma, 2-3 mm in thickness, were fixed in 10% phosphate-buffered neutral formalin for 1-3 hr. The fixed slices were washed in phosphate-buffered saline (PBS) overnight and frozen-sectioned at thicknesses of 50 μ. The sections were reacted with the acid phosphatase-labeled anti-EBM for 2-3 days in a cold room (4°C) with gentle agitation, washed in PBS overnight, and washed in 0.05 M acetate buffer at pH 5.2 for 2 hr. The sections were stained in Gomori's medium (5) for 30 min at 37°C, washed in acetate buffer for 1 hr, postfixed at 1.2% Veronal-buffered glutaraldehyde for 1 hr, and washed in 0.25 M sucrose for 1 hr. The sections then were transferred to 2% OsO₄ buffered with s-collidine (3), dehydrated in graded alcohols and embedded in Epon 812 (14). Control sections were reacted with the fraction of the conjugates that had been reacted with antigen prior to their application. Thin sections were cut on a LKB Ultrotome, stained with lead (10), and examined in an RCA EMU-3F.

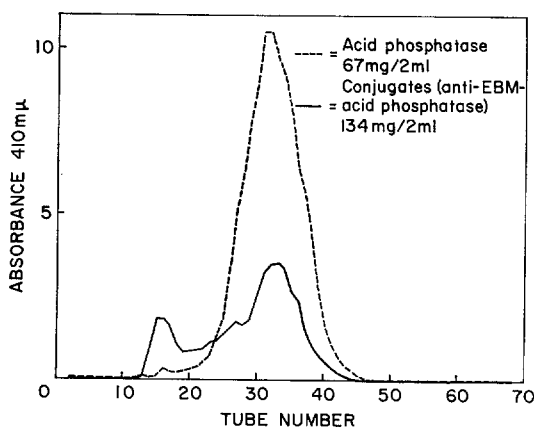For a check on the materials before the remaining

FIGURE 2 Acid phosphatase activity of the fractions of acid phosphatase-anti-EBM conjugate and unconjugated acid phosphatase shown in Fig. 1.

tissue sections were processed for electron microscopy, some sections were withdrawn prior to postfixation in glutaraldehyde and placed in 1% ammonium sulfide solution for 1-2 min; then the sections were observed in a light microscope.

When the unfractionated reaction mixture was used, the penetration of the conjugate was limited to only two or three cell layers from the surfaces of the tissues, as determined by sectioning the tissue slices in a cryostat and staining for the enzyme. However, the antibody was localized throughout the tissue sections, as determined by staining the sections with fluorescein-labeled anti-rabbit gamma globulin. It is postulated that the free antibody in the reaction mixture penetrated through tissues faster than the conjugate and that it occupied and blocked the antigenic sites before the arrival of the conjugates. For reasons not altogether clear, the best penetration of the conjugate through tissue slices was obtained when the fractions obtained from the columns were used.

To test nonspecific affinity of acid phosphatase to tissues, sections were reacted with the conjugate, a mixture of acid phosphatase and rabbit gamma globulin without the conjugation reaction, with a solution of acid phosphatase, or with a solution of rabbit gamma globulin. Other sections that were reacted with the conjugate, the mixture, or the solution of rabbit gamma globulin were stained with fluorescein-labeled sheep anti-rabbit gamma globulin. Other sections that were reacted with the conjugates, the mixture, or the solution of acid phosphatase were stained histochemically for the enzyme. To test nonspecific binding of lead in the nucleus (2, 5, 18), some of the sections were incubated in Gomori's medium from which the substrate, β-glycerophosphate, was omitted.

Attempts to purify the acid phosphatase-conjugated antibody from the uncoupled reagents in the reaction mixture by means of the thin layer and the gel electrophoresis were unsuccessful. The enzymatic activity of the conjugates was lost rapidly during the procedure.

Preparation of Peroxidase-Labeled Antibodies

In the preliminary studies, active peroxidase-antibody conjugates were obtained only when FNPS was used as the bifunctional reagent. With various concentrations of FNPS, optimal conditions for the conjugation reaction were established by varying the concentrations of peroxidase and antibodies, the pH of the reaction mixture, the buffer strength, and the duration of the reaction. The effectiveness of the conjugation was tested immunohistochemically.

Crude, Type II or Type VI peroxidase (from Sigma Chemical Co.) may be conjugated to the antibodies. An increase in the concentration of peroxidase over 2.5% did not increase the yield; however, a decrease in the concentration resulted in a lesser yield.

Maximum yields were obtained when the reaction was allowed to continue for 4 hr at a pH between 10 and 10.5. Prolonging the time did not improve the yield. When the reaction took place above pH 11 or below 9.5, the yield was diminished; when the reaction took place at pH 8 or below, conjugation did not occur. An increase in the concentration of carbonate or FNPS did not improve the yield. Hence, all subsequent conjugations were carried out by the routine method indicated below. Following the conjugation reaction, unreacted peroxidase was removed by precipitation in 50% ammonium sulfate solution.

The conjugates were prepared routinely in the following manner: to 50 mg of horseradish peroxidase and 50 mg of gamma globulin dissolved in 2 ml of 0.5 M cold carbonate buffer at pH 10 was added 0.25 ml of 0.5% FNPS in acetone; the mixture was agitated gently for 6 hr at 4°C and was dialyzed against PBS overnight; a precipitate was removed by centrifugation.

In order to isolate the peroxidase-antibody conjugate, the dialyzed material was fractionated in a column of Bio-Gel P-300 and eluted by PBS. Each fraction was analyzed for its protein content spectrophotometrically at 280 mμ. The peroxidase activity of each fraction was determined with pyrogallol (15) as substrate, and the peroxidase content of each fraction was determined spectrophotometrically at 403 mμ.

When the reaction mixture was fractionated, three protein peaks were present: at tubes No. 23 (void volume of the column), 35, and 50 (Fig. 3). These peaks represented denatured protein, unreacted antibody, and unreacted peroxidase, respectively. The staining ability was found in tubes No. 28–36, with a maximum staining ability at tube No. 31.

Since peroxidase remains in suspension and gamma globulin precipitates in a solution of 50% saturated ammonium sulfate (31), the unreacted peroxidase was separated from conjugated and unconjugated gamma globulin by precipitating the latter in ammonium sulfate solution. The precipitate was resuspended in PBS, and the ammonium sulfate was removed by dialysis against PBS. The solution was fractionated through a column; the peroxidase peak at tube No. 50 was removed completely (Fig. 3). However, the staining ability of the fractions between tubes No. 22 and 30 was unchanged. These fractions which contained the immunologically and enzymatically active conjugates were employed in the localization of FBM.

Peroxidase-Labeled Antibodies for the Localization of Tissue Antigens

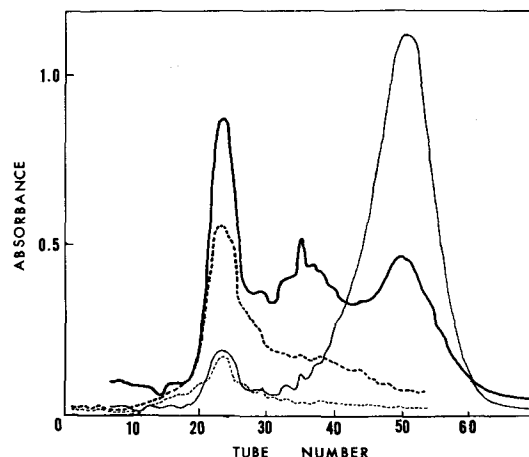LIGHT MICROSCOPIC PROCEDURE : For the light microscopic staining of tissues, 6-μ thick frozen

FIGURE 3   Fraction patterns of peroxidase-gamma globulin conjugates before and after ammonium sulfate precipitation. Broad solid line shows 280 mμ absorption before the precipitation; broad broken line, 280 mμ absorption after the precipitation; narrow solid line, 403 mμ absorption before the precipitation; narrow broken line, 403 mμ absorption after the precipitation. Column height: 41 cm. Volume per fraction: 2.9 ml.

sections of mouse spleen, kidney, or parietal yolk sac carcinoma were fixed briefly in cold acetone or in 10% phosphate-buffered neutral formalin, washed in PBS, and reacted initially for 30 min with either rabbit gamma globulin containing antibody, or, in the controls, with the antibody which had been absorbed with its antigen. After washing three times in PBS, the sections were reacted with the peroxidase-labeled anti-rabbit gamma globulin for 30 min and washed three times in PBS.

Even though the direct technique was successful, it was more practical to use the indirect method perfected for light microscopy (4). The sections that were reacted with the peroxidase-labeled antibodies were stained for the enzyme by the method of Karnovsky (8). The sections were incubated at room temperature, for 10–30 min, in a solution composed of 75 mg of 3,3'-diaminobenzidine and 0.001% peroxide in 100 ml of 0.05 M tris buffer, pH 7.6, washed in the tris buffer, osmicated in 2% OsO$_4$ in distilled water, washed, dehydrated, cleared in xylol and mounted. The reaction products were dark brown to black and readily recognized.

ELECTRON MICROSCOPIC PROCEDURE : Parietal yolk sac carcinoma was fixed in 10% phosphate-buffered neutral formalin for 1 hr in a cold room (4°C). The tissue was sectioned at about 50 μ in thickness with a Smith and Farquhar Tissue Sectioner (28) and further fixed for 2 hr in the formalin fixative. The tissue sections were washed in PBS overnight,

reacted with either peroxidase-labeled anti-EBM or peroxidase-labeled anti-EBM absorbed with its antigen (for control) for 2–3 days at 4°C with gentle agitation, washed in PBS overnight, fixed in 5% glutaraldehyde for 1 hr, washed in PBS overnight, and stained for peroxidase by Karnovsky's method (8, 11). The stained sections were washed three times in distilled water, osmicated, dehydrated, and embedded in Epon (14). Thin sections were examined in an electron microscope without further staining.

When the reacted tissue sections were incubated in Karnovsky's peroxidase medium without postfixation in glutaraldehyde, the enzymatic activity or the reaction product tended to damage the ultrastructure of the antigenic sites in neighboring areas. Postfixation in 5% glutaraldehyde prior to incubation reduced the damage and did not interfere with the peroxidase activity.

RESULTS

Light Microscopic Observations of Tissue Sections Reacted with Acid Phosphatase-Labeled Anti-EBM

When acid phosphatase-labeled anti-EBM was reacted with tissue sections of the parietal yolk sac carcinoma and was followed by the histochemical reaction for acid phosphatase, reaction

products were deposited on the EBM only (Fig. 4 *a*). Conjugate absorbed with EBM prior to its application failed to stain these tissues, indicating the immunologic specificity of the reaction (Fig. 4 *b*).

The nuclei of both control and experimental preparations stained. The nuclei did not stain when the substrate (β-glycerophosphate) was withdrawn from Gomori's medium, consequently, the nuclear staining did not represent nonspecific precipitation of lead nitrate. The observation that acid phosphatase alone would bind to nuclei indicates that the mechanism of nuclear staining was the result of this unexplained affinity of the enzyme which resulted in nonimmunologic binding of the conjugate. The presence of enzyme-labeled antibody in the nuclei was determined by the indirect technique with fluorescein-labeled anti-rabbit gamma globulin.

Electron Microscopic Observations of Tissue Sections Reacted with Acid Phosphatase-Labeled Anti-EBM

The crystalline reaction products of the enzyme were precipitated on the extracellular basement membrane material of parietal yolk sac carcinoma
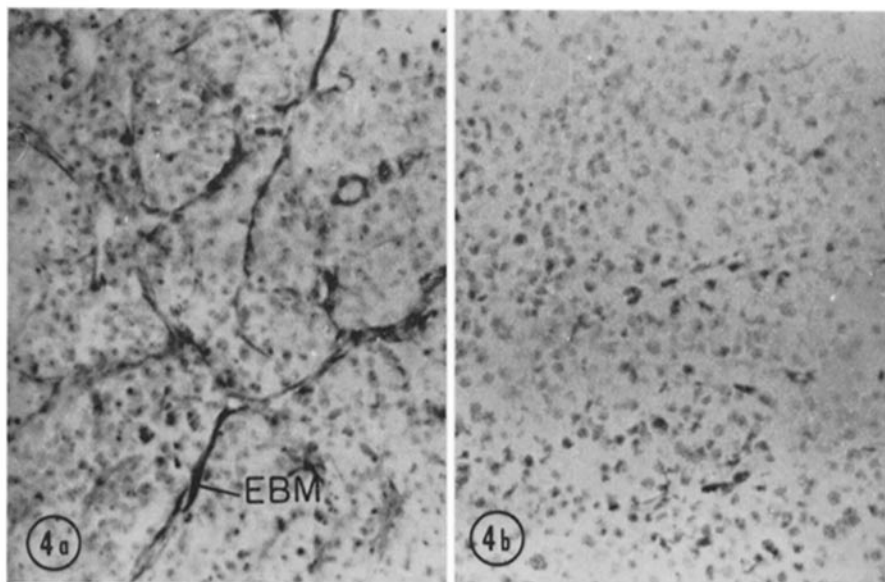

FIGURE 4 Mouse parietal yolk sac carcinoma reacted with acid phosphatase-labeled anti-EBM (*a*) and with acid phosphatase-labeled anti-EBM absorbed with EBM prior to its application (*b*), and stained for the enzyme. The black reaction products are present at the sites of EBM in the tumor. The control failed to stain the EBM. Nuclei of both preparations are stained. × 150.

(Fig. 5). The endoplasmic reticulum also was labeled with the reaction products (Fig. 6). This was to be expected since it had been shown previously that the carcinoma synthesized EBM in vivo and in vitro, and that ferritin-labeled anti-EBM localized in the endoplasmic reticulum (20).

As in the case of the light microscopic observation, the nuclei also were stained with the reaction product (Fig. 7). In the control sections, the nuclei were stained whereas the EBM and cytoplasm were virtually free from reaction product.

Light Microscopic Observations of Tissue Sections Reacted with Peroxidase-Labeled Antibody

In the sections of parietal yolk sac carcinoma, the reaction products were localized on the EBM when the sections were reacted in either the direct or indirect technique with anti-EBM (Fig. 8 *a*). Some of the carcinoma cells contained reaction products which presumably represented sites of synthesis of the antigen. The sections reacted with either anti-collagen (Fig. 8 *b*) or anti-EBM absorbed with EBM (Fig. 8 *c*) failed to stain.

On the other hand, when the sections of spleen were reacted with peroxidase-labeled anti-collagen, the reticulin was stained (Fig. 9 *b*). If the sections were reacted with anti-EBM (Fig. 9 *a*) or with anti-collagen absorbed with collagen (Fig. 9 *c*), the reticulin failed to stain. Endogenous peroxidase in leukocytes (1) was stained in each of these preparations.

Electron Microscopic Observations of Tissue Sections Reacted with Peroxidase-Labeled Anti-EBM

As was the case with ferritin-labeled anti-EBM (20) and acid phosphatase-labeled anti-EBM, the extracellular basement membrane (Fig. 10) and the material contained within the endoplasmic reticulum were stained densely (Fig. 11). The reaction products were electron-opaque and noncrystalline with a fine globular appearance, whereas in the control section no reaction product was observed (Fig. 12). This confirmed the presence of EBM in each of these sites and indicated the specificity of the peroxidase-labeled antibody method.

DISCUSSION

The recent development of the ultrastructural localization of enzymes and of organic and inorganic substances has advanced the understanding of cell structure and function. Yet the majority of macromolecules which lack unique morphological features or distinct chemical reactions are unrecognizable ultrastructurally. The immunohistochemical principle developed by Coons and his colleagues (4) and adapted to electron microscopy by several investigators (19, 27, 29, 30) has been used successfully to localize macromolecules, provided that a highly specific antibody against the macromolecules were obtained.

Success with the electron microscopic immunohistochemical technique depends upon the following: electron-scattering ability of the label conjugated to antibody; conjugation of label and antibody by stable covalent linkages without altering the nature of the antibody; the small molecular size of the label to avoid steric effects that might interfere with the mobility of the antibody in inter- and intracellular spaces; and stability of the label under the adverse physical and chemical conditions presented by electron microscopy. Ferritin (27) and heavy metals (19, 29, 30) have been useful labels for antibodies, although they have failed to satisfy one or more of the above criteria.

FIGURE 5   Mouse parietal yolk sac carcinoma stained with acid phosphatase-labeled anti-EBM. The reaction products of the enzyme are present in the extracellular EBM as well as in the EBM in the cisternae of endoplasmic reticulum (*ER*). The nucleus (*N*) also is stained. *M*, mitochondria. $\times$ 25,000.

FIGURE 6   Cytoplasm of the mouse parietal yolk sac carcinoma stained with acid phosphatase-labeled anti-EBM. The EBM in the endoplasmic reticulum is stained. Mitochondria (*M*) and other areas of cytoplasm are not stained. $\times$ 23,000.

FIGURE 7   Control. Mouse parietal yolk sac carcinoma stained with acid phosphatase-labeled anti-EBM absorbed with EBM prior to its application. Extracellular EBM and EBM in the endoplasmic reticulum (*ER*) are not stained. The cell nucleus (*N*) is stained. *M*, mitochondria. $\times$ 20,000.
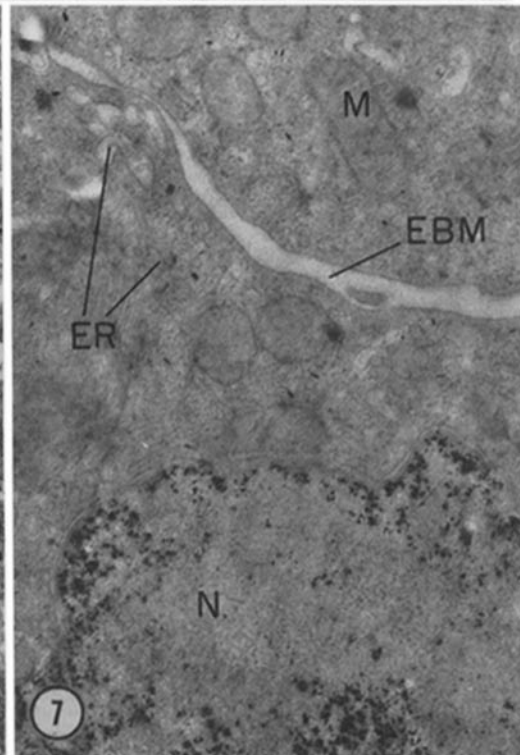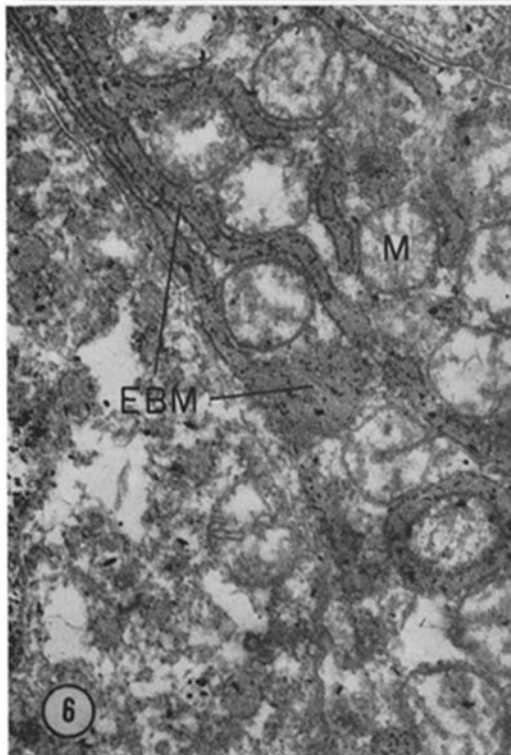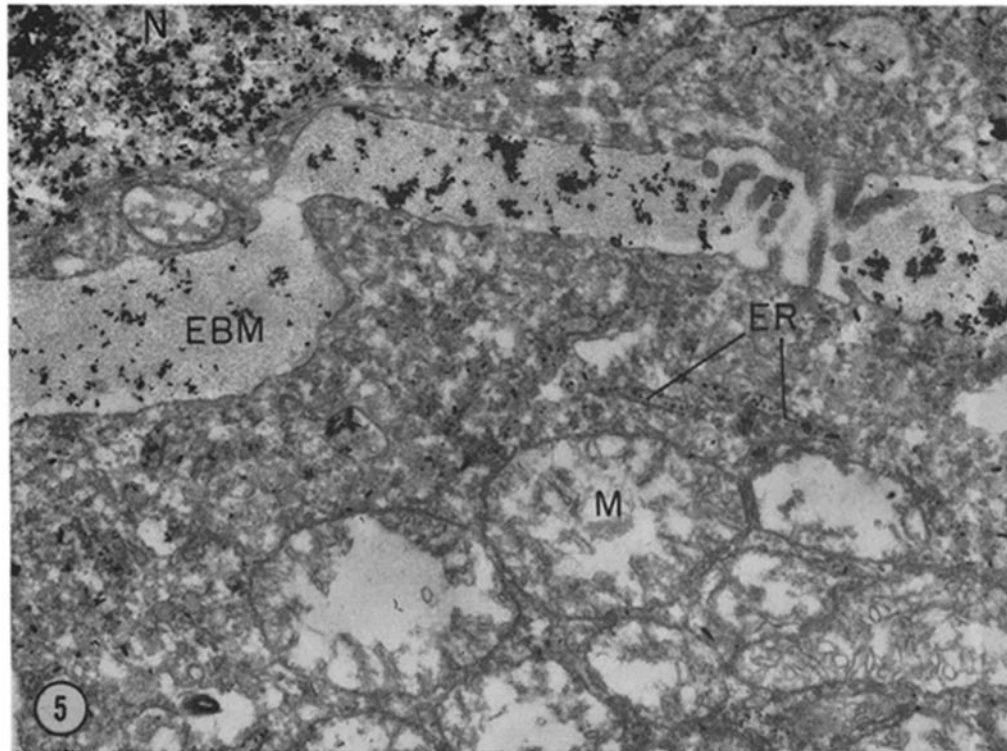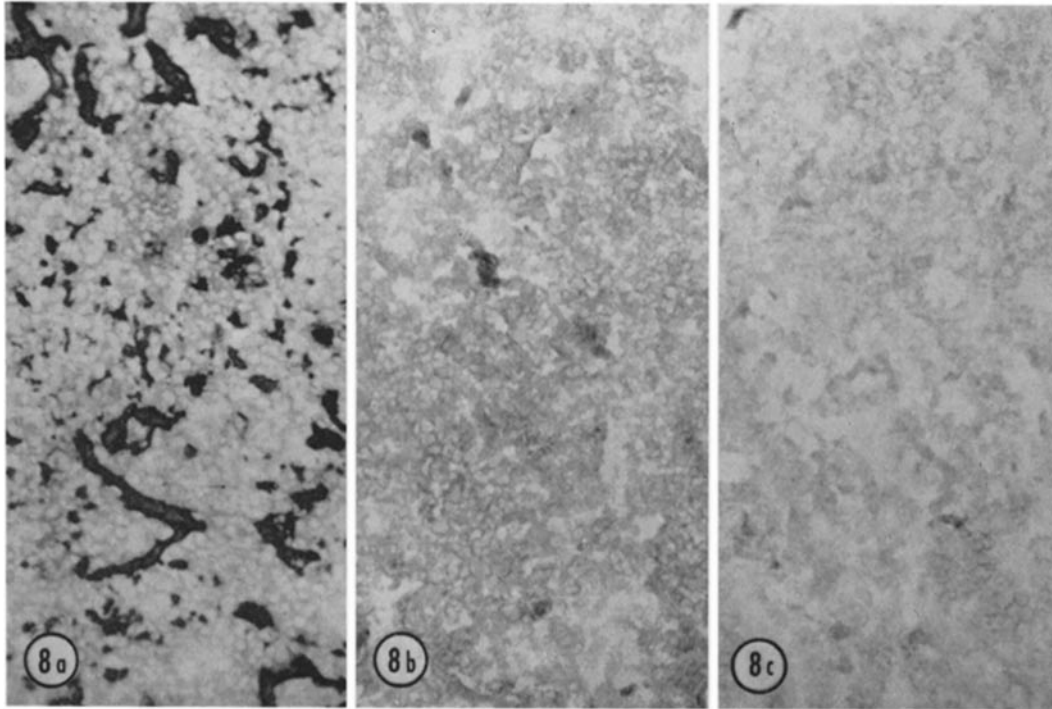
FIGURE 8 *a*, *b*, and *c* are sections of mouse parietal yolk sac carcinoma stained respectively with anti-EBM, anti-collagen, and anti-EBM that has been absorbed with EBM. The sections then were stained with peroxidase-labeled sheep anti-rabbit gamma globulin and finally stained histochemically for peroxidase. The black precipitates are present at the sites of EBM in the tumor (*a*). Neither anti-collagen (*b*) nor the control (*c*) stained EBM. × 250.

Ferritin with a molecular weight in excess of 650,000 (9) and a diameter of about 120 A (25) is identifiable with certainty, but its large size precludes easy penetration of tissue. Antibody labeled with heavy metal penetrates tissues easily, but in the past the increase in contrast provided at the sites of antigen-antibody reaction has been insufficient to be useful (22, 26). More recently, by a complex method, contrast has been improved (29).

Enzymes as labels for antibodies offer several unique advantages. Many enzymes are of small molecular weight, i.e. that of peroxidase is about 40,000 (12), and the conjugate can be expected to penetrate through tissues more easily than ferritin-labeled antibody. The enzyme and antibody are conjugated through stable covalent linkages, and the reaction products of the histochemical reaction are insoluble in organic solvents and stable under the electron beam. In addition, enzymes are not consumed in the histochemical

reaction and many molecules of electron-opaque reaction product are deposited at the antigenic site. This amplifying ability of the enzyme makes the method extremely sensitive. The intensity of staining or the size of reaction product may be controlled by varying the duration of incubation.

Ideally, the enzyme should be a kind which either does not exist in the material being studied or should be in well-defined situations so that the sites of antigen may not be confused with those of endogenous enzyme.

In the present study, wheat germ acid phosphatase and horseradish peroxidase were employed since they were obtainable from commercial firms and were stainable histochemically at light (2, 18) and electron microscopic levels (6, 11), and since the endogenous localizations are limited to certain well-defined sites (6, 18).

Acid phosphatase was the first enzyme to be conjugated to antibody. Although the conjugates penetrate tissues well and have easily defined
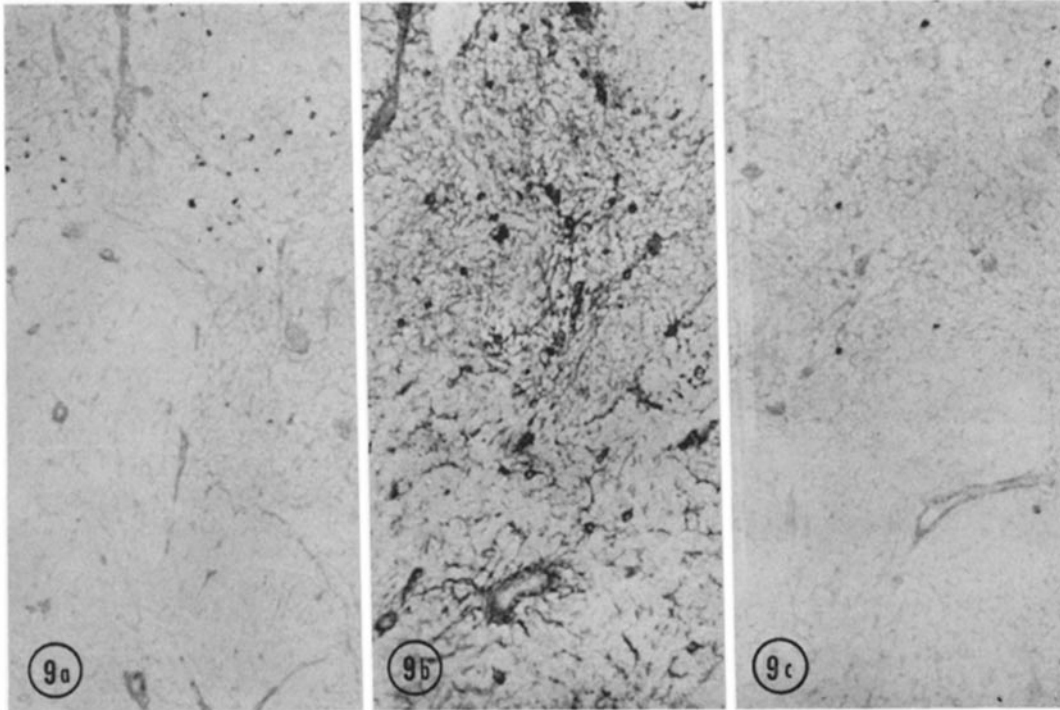
FIGURE 9 a, b, and c are sections of mouse spleen stained respectively with anti-EBM, anti-collagen, and normal rabbit serum and then stained with peroxidase-labeled sheep anti-rabbit gamma globulin. In these sections endogenous peroxidase in leukocytes is stained. Only the section stained with anti-collagen (b) stained the reticulin of the spleen. Anti-EBM (a) and normal rabbit serum (c) failed to stain the reticulin. × 250.

crystalline reaction products under the electron microscope, the method of preparing the conjugates was exceedingly unreliable. Inactive conjugates were obtained in three out of five attempts, and the conjugates that were active were unstable and deteriorated within a week. Moreover, the affinity of the enzyme for the nucleus makes it impossible to localize antigens in the nucleus. Conversely, the method for conjugating peroxidase and antibody lacked the difficulties associated with that for conjugating acid phosphatase and antibody, and resulted in a conjugate which was stable in the refrigerator for a month without apparent deterioration and which could be kept lyophilized for an indefinite time.

Because the reaction products of some enzymes differ in their morphological appearance (amorphous, or crystals of varying structure), theoretically it should be possible to localize several antigens simultaneously by using antibodies each of which is labeled with a different enzyme.

Unlike ferritin-labeled antibody, both acid phosphatase-labeled and peroxidase-labeled antibodies penetrate through tissue easily. The reaction products of the enzymes were localized at the extracellular as well as the intracellular antigenic sites, indicating that the enzyme-labeled antibody had the same specificity as the ferritin-labeled antibody.

It has been demonstrated in the past that the light microscopic method of localizing antigen is a useful preliminary to the ultrastructural method. The logistics of a system can be worked out with the former method which offers considerable saving in time and money compared with the electron microscopic method alone. The enzyme-labeled antibody method has been especially useful in the preliminary experiments with the light microscope. In specificity and sensitivity the method compares favorably with the fluorescein-antibody method. There is no inter-

fering autofluorescence, the preparations are permanent, the amount of photography necessary is reduced, and the specimens can be examined with ordinary microscopes. The fluorescein-antibody technique is a most useful one, but the advantages of the enzyme-labeled antibody method render it more convenient.

In conclusion, enzymes and antibodies may be conjugated and still retain both enzymatic and immunologic activities. The conjugates are spe-

cific and may be used to demonstrate antigenic sites at both light and electron microscopic levels.

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FIGURE 10 Mouse parietal yolk sac carcinoma which produces and secretes EBM was reacted with peroxidase-labeled anti-EBM and stained for the enzyme with Karnovsky's method. Extracellular EBM as well as EBM in cisternae of endoplasmic reticulum (ER) are stained densely. Cell nucleus (N) and mitochondria (M) are free from the staining. $\times$ 20,000.

FIGURE 11 Cytoplasm of the carcinoma treated in the same manner as in Fig. 10. The reaction products of the enzyme are localized on the EBM within the cisternae of endoplasmic reticulum; mitochondria (M) and cytoplasm are free from the staining. $\times$ 20,000.

FIGURE 12 Control. Cytoplasm of the carcinoma which has been reacted with the peroxidase-labeled anti-EBM absorbed with EBM prior to its application and then stained for the enzyme with Karnovsky's method. All staining is abolished. M, mitochondria. $\times$ 20,000.
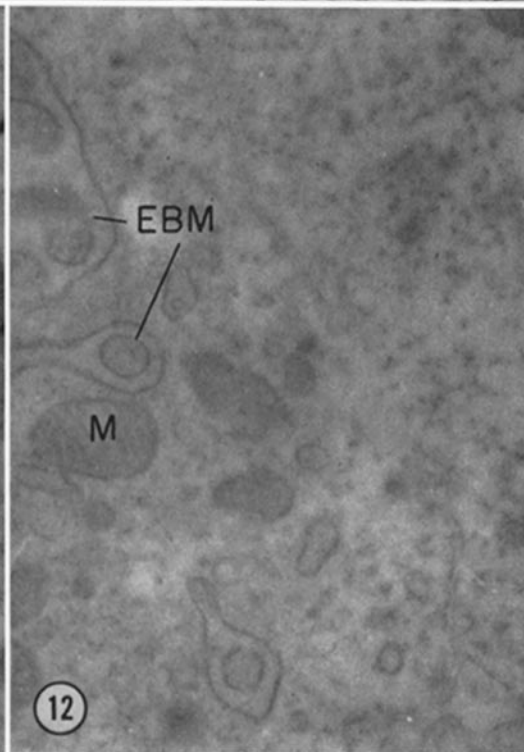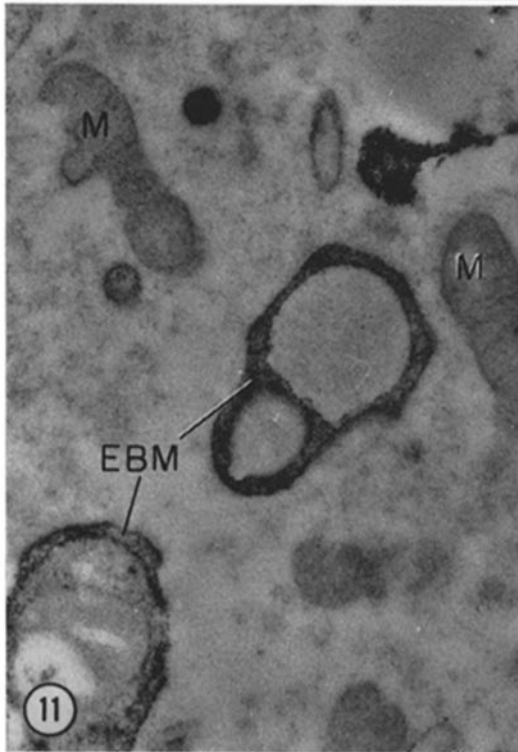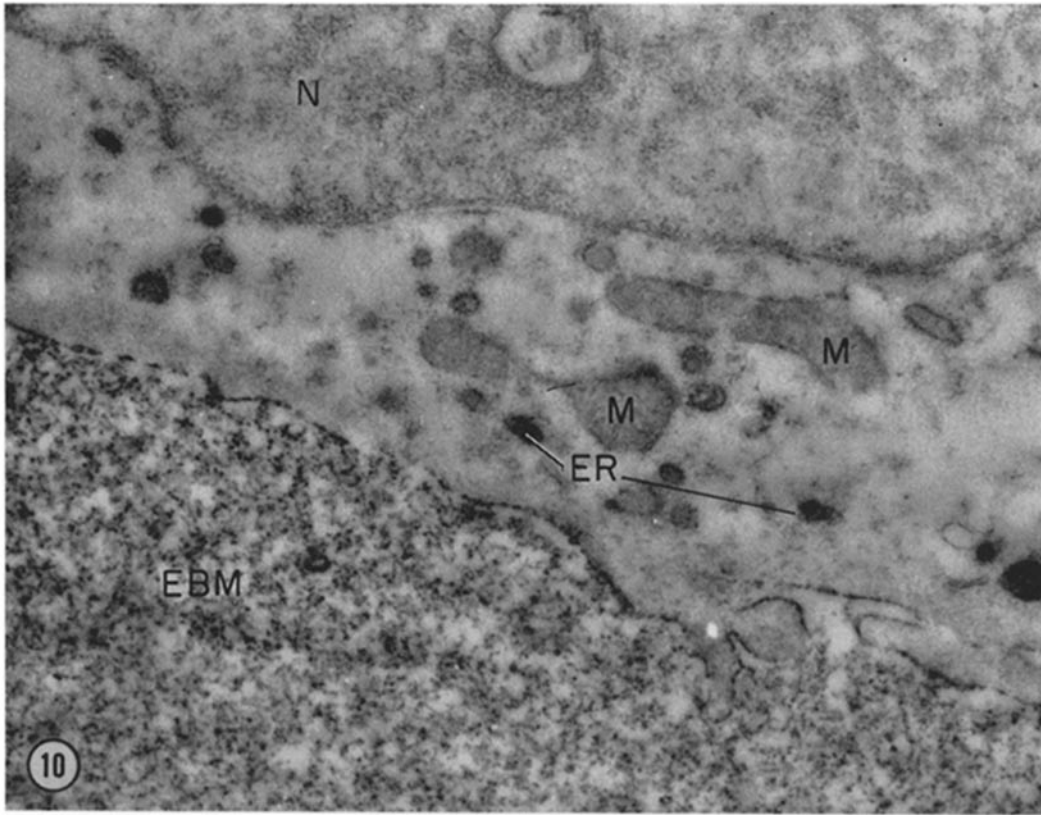
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