APPLICATION OF IMMUNOSTAINING AND CORRELATED CYTOCHEMICAL METHODS IN SEVERAL SITES

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A number of techniques have been developed recently for localization of specific antigens at the ultrastructural level by immunostaining. The processing techniques in general utilize formalin or glutaraldehyde fixation, subsequent immunostaining of cryostat sections and epoxy embedding or employ fixation and embedding followed by immunostaining of ultrathin sections. Several different techniques have been introduced for ultrastructural immunostaining of cell antigens. These include conjugates of antibody with ferritin, labeling of antibody with heavy metals and conjugates of whole antibody or Fab fragments of gamma globulin with enzymes such as phosphatase, horseradish peroxidase or cytochrome C. In addition, bridge immunostaining techniques have been developed which bind the antibody to the tissue antigen through antibody-antigen reactions and thus avoid alteration of the immunoreactivity of the antibody that might result from conjugation of the enzyme to a labeling macromolecule. These bridge methods include hybrid antibodies and immunoglobulin-enzyme bridge techniques. An example of the latter procedure involves initial staining with specific antibody prepared in rabbits and subsequent staining in order with goat antirabbit globulin, antihorseradish peroxidase prepared in rabbits, and horseradish peroxidase prior to incubation in the substrate medium for the horseradish peroxidase. The antibody-enzyme conjugates and the bridge procedures provide immunostaining at the ultrastructural level through enzymatic buildup of reaction product that is electron opaque.

The immunoglobulin peroxidase bridge procedure has been applied in our laboratory at the light microscope level to visualize hormones in various cells of the human adenohypophysis. The results agree in general with those obtained with the Nakane antibody peroxidase conjugate applied to rat pituitary. Staining with specific antisera has localized ACTH and μ -MSH in the same cell type in the human parts distalis, and these cells are now referred to as the melanocorticotrophic cells. Antisera to follicle stimulating hormone and luteinizing hormone have visualized these two hormones in the same gonadotropic cells and antiserum to thyroid stimulating hormone has visualized the third type of mucoid cell—the thyrotropic cell.

The immunoglobulin peroxidase bridge procedure applied at the ultrastructural level to visualization of human chorionic gonadotropin (HCG) has localized this hormone in the granular reticulum at its site of biosynthesis and on the cell surface in the syncytiotrophoblast. Cytotrophoblasts in this organ lack immunoreactivity for HCG. The failure of the rather scant Golgi complexes and various types of cytoplasmic dense bodies in the syncytium to immunostain for HCG casts doubt on the previously indicated role of these dense bodies in storage and secretion of the

hormone. Their acid phosphatase activity indicates these dense bodies are lysosomes. The possibility remains that in this site hormone may be secreted directly from the granular reticulum.

Staining with dialyzed iron visualizes acid mucosubstance ultrastructurally and shows hyaluronic acid (i.e., hyaluronidase labile, nonsulfated mucosubstance at the light microscope level) on the surface of the placenta. Staining for glycoprotein by the Avrameas Concanavalin A-horseradish peroxidase (Con-A-HRP) method also visualizes ultrastructurally a heavy coat of reactive material on the placental surface. Since hyaluronidase fails to eliminate the Con-A-HRP staining on the surface of the placenta the latter technique demonstrates a different component from that with dialyzed iron affinity. Questions remain as to whether the glycoprotein with Con-A-HRP reactivity may be HCG or a third component in this site, and whether these substances may occupy different loci on the surface as indicated by the apparent periodicity of the staining.

The immunoglobulin peroxidase bridge method applied at the ultrastructural level to visualization of the Terasaki-type human lymphocyte antigens has differentiated lymphocytes on the basis of antigen type and in contradistinction to earlier reports, has indicated the presence of the antigen uniformly over the lymphocyte surface. Immunostaining with a rabbit antiserum to calf lymphocytes has shown similar uniform distribution over the calf lymphocyte surface of the antigens against which this antiserum was generated. Rh+ erythrocytes immunostained for D antigen have revealed dense deposits of peroxidase reaction product spaced at about 250 Å in confirmation of previous studies with ferritin labeled antibody.

The Con-A-HRP technique also yields electron opaque deposits distributed diffusely over the surface of lymphocytes as well as other blood cell types. The glycoprotein stained by this method appears to differ in various cell types as evidenced by variable susceptibility of the staining in different cells to prior blockage by acetylation. Current investigations undertake to determine the relation between the sites on the lymphocyte surfaces stained with the Con-A-HRP technique and those visualized immunocytochemically with antisera to lymphocyte antigens.

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REFERENCES

1. Coons, A. H.: Fluorescent antibody methods. In *General Cytochemical Methods*, edited by J. F. Danielli, Vol. 1, Academic Press, New York, p. 399, 1958.
2. Dreskin, R. B., Spicer, S. S. and Greene, W. B.: Ultrastructural localization of chorionic gonadotropin in human term placenta. *J. Histochem. Cytochem.* 18; 862, 1970.
3. Hämmerling, U., Aoki, T., DeHarven, E., Boyse, E. A. and Old, L. J.: Use of hybrid antibody with antigamma G and antiferritin specificities in locating cell surface antigens by EM. *J. Exp. Med.* 128; 1461, 1968.
4. Kawai, Y., and Nakane, P. K.: Localization of tissue antigens on the ultrathin sections with peroxidase-labeled antibody method. *J. Histochem. Cytochem.* 18; 161, 1970.
5. Kraehenbuhl, J. P., DeGrandi, P. B. and Campiche, M. A.: Ultrastructural localization of intracellular antigen using enzyme-labeled antibody fragments. *J. Cell Biol.* 50; 432, 1971.
6. Mason, T. E., Phifer, R. F., Spicer, S. S., Swallow, R. A. and Dreskin, R. B.: An immunoglobulin-enzyme bridge method for localizing tissue antigens. *J. Histochem. Cytochem.* 17; 563, 1969.

7. Nakane, P. K. and Pierce, G. B.: Enzyme-labeled antibody for the light and electron microscopic localization of tissue antigens. *J. Cell Biol.* 33; 307, 1967.
8. Phifer, R. F., Midgley, A. R. and Spicer, S. S.: Immunohistologic and histologic evidence that follicle-stimulating hormone and luteinizing hormone are present in the same cell type in the human pars distalis. *J. Clin. Endocrinol. Metab.* In press, 1972.
9. Phifer, R. F., Spicer, S. S. and Orth, D. N.: Specific demonstration of the human hypophyseal cells which produce adrenocorticotrophic hormone. *J. Clin. Endocrinol. Metabol.* 31; 347, 1970.
10. Singer, S. J. and McLean, J. D.: Ferritin antibody conjugates as stains for electron microscopy. *J. Lab. Invest.* 12; 1002, 1963.
11. Sternberger, L.: Electron microscope immunocytochemistry: A review. *J. Histochem. Cytochem.* 15; 137, 1967.
12. Sternberger, L. A., Hardy, P. H., Jr., Cuculis, J. J. and Meyer, H. G.: The unlabeled antibody enzyme method of immunohistochemistry. *J. Histochem. Cytochem.* 18; 315, 1970.
13. Willingham, M. C., Spicer, S. S. and Graber, C. D.: Immunocytochemical labeling of calf and human lymphocyte surface antigens. *Lab. Invest.* 25; 211, 1971.

FIG. 1. A portion of syncytiotrophoblast displays electron opaque deposits of peroxidase reaction product indicative of immunostaining for HCG. These deposits, covering the outer or maternal surface of the apical plasma membrane and filling cisternae of ergastoplasm (e), have a finely particulate character. The deposits of reaction product appear to show a fine periodicity on the surface of the apical plasma membrane and tend to concentrate on the luminal surface of the surface of the rough endoplasmic reticulum. Caviolae (short arrows) at the apical plasma membrane are immunostained. Thick annular profiles of immunoreactive material bridging between microvilli (*) are possibly related to ghostlike outlines observed in morphologic preparations. The outer surface of the folded, basal plasma membrane discloses light immunostaining. Peroxidase reaction product is not evident in profiles presumed to represent the small variety of cytoplasmic granules (long arrow) or in Golgi elements (g), mitochondria (m) or basement membrane (bm). The cryostat section was stained with anti-HCG as the first step in the immunoglobulin-peroxidase bridge. The thin section was unstained. $\times 20,000$.

FIG. 2. The syncytiotrophoblast (S) reveals immunostaining for HCG on the surface and in ergastoplasm (e), but the underlying cytotrophoblast lacks such reactivity in any of its structures including the nucleus (n), scant elements of rough endoplasmic reticulum (arrows), Golgi elements (g), mitochondria (m) or hyaloplasm. Immunostained cryostat section, unstained thin section. $\times 13,300$.

FIG. 3. In contrast to the immunostained cisternae of the rough endoplasmic reticulum (e), the lamellae and vesicles of the Golgi complex (g), a cytoplasmic granule of the small variety (arrow) and mitochondria (m) lack dense reaction product indicative of immunoreactivity with anti-HCG. Immunostained cryostat section, unstained thin section. $\times 21,300$

FIG. 4. The maternal surface of the apical plasma membrane and ergastoplasmic cisternae (e) immunostain for HCG. A large cytoplasmic granule interpreted as a multivesicular body (arrow) lacks immunoreactivity. Immunostained cryostat section, unstained thin section. $\times 12,300$.

FIG. 5. In this control preparation the apical plasma membrane covering the microvilli (mv) and the ergastoplasmic cisternae (e) lack deposits of peroxidase reaction product. Some large cytoplasmic granules are the only structures that contain dense deposits indicative of intrinsic peroxidase. Other similar structures (arrows) reveal light or no deposits. The cryostat section was stained with a control immunoglobulin-peroxidase bridge procedure in which nonimmune rabbit serum replaced anti-HCG rabbit serum as the first component. Unstained thin section. $\times 12,300$.

FIG. 6. A calf lymphocyte from a control preparation in which the first step (i.e., the rabbit antiserum to calf lymphocytes) in the immunoglobulin peroxidase bridge was omitted. $\times 4,700$.

FIG. 7. Ultrastructural immunostaining of calf lymphocytes with rabbit anticalf lymphocyte serum as the first component of the immunoglobulin-peroxidase bridge. The calf lymphocyte discloses continuity of deposit over the surface without large unlabeled areas. $\times 9,400$.

FIG. 8. Human peripheral blood. Rh+ erythrocytes immunostained for D antigen with the peroxidase bridge method. Dense deposits of peroxidase reaction product (arrows) spaced at intervals of about 250 m μ lie on the surfaces of two erythrocytes. $\times 19,000$.