**PRE-EMBEDDING IMMUNOELECTRON MICROSCOPY BY
PEROXIDASE-LABELED ANTIBODY METHOD
—APPLICATIONS TO ENDOCRINOLOGY—**

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Pre-embedding immunoelectron microscopy has demonstrated the ultra-structural localization of antigens in various intracellular organelles, including cisternae of rough endoplasmic reticula, peri-nuclear spaces, Golgi saccules, cytosol, secretory granules and mitochondria.

In this report, major staining procedures of this method are briefly described and its applications to endocrinology are demonstrated.

For the last decade, the immunoperoxidase method has become a common technique in immunohistochemistry and recently many investigators have required the observation of bioactive substances at the electron microscopic level. On the application of this immuno-peroxidase method at the electron microscopic level, two different methods (15, 16) have been used, 1) pre-embedding method applied on 4–10 μm frozen sections (11, 15, 16) and, 2) post-embedding method applied on ultrathin sections (4, 16, 17). In our laboratory, the pre-embedding method has been preferred to localize the various substances at the ultrastructural level, because of the following two major reasons; 1) constant and more sensitive staining can be expected by better preservation of the antigenicity and, 2) very fine localization at any subcellular organelles, i.e. cell membrane, cytosol, Golgi saccules, secretory granules, or mitochondria, can be obtained with this method (16).

Among various scientific fields, the endocrinology has been applying the immunohistochemical technique extensively. In fact, Nakane applied his technique first to the pituitary gland to localize anterior pituitary hormones (10). Since then, many investigators have localized various hormones in the various endocrine tissues, mostly at the light microscopic level (10, 12, 13, 17, 18, 19). By applying the immunoelectron microscopic technique, further detailed cytologic observations of the endocrine cells have been attempted (11, 14). In our laboratory, as applications of immunoelectron microscopy to endocrinology, this technique has been used in two aspects, i.e., 1) localization of hormones and, 2) localization of enzymes related to hormone production and metabolism.

In this context, we have been studying the ultrastructural localization of peptide hormones in the rat and human pituitary glands (21, 22), the enzymes related to adrenocortical steroidogenesis (cytochrome P450, adrenodoxin, adrenodoxin reductase) (3, 6, 7) and the enzyme related to the adrenocortical lipid metabolism

(glutathione peroxidase) (8, 9). These immunoelectron microscopic observations can be expected to be extremely useful tools to study the secretory pathways of peptide hormones or functional status of steroidogenic endocrine cells.

In this report, major procedures and technical problems of immunoelectron microscopic staining applied in our laboratory are briefly described and some applications of this technique to endocrinology are demonstrated.

1. TECHNIQUES AND PROCEDURES

Among various immunoelectron microscopic techniques for endocrine organs, the following two methods are most commonly used.

- (1) Peroxidase-labeled antibody method (Nakane) (10)
- (2) Peroxidase antiperoxidase method (Sternberger) (23)

The main difference between these two methods is that, in the labeled antibody method, smaller fragments of IgG such as Fab' or Fab fragments can be used in order to enhance the penetration of the labeled antibody into the frozen sections. In the peroxidase antiperoxidase (PAP) method, such smaller fragments as Fab' or Fab theoretically can never be used. And if PAP complex is used, this large molecule might not be able to penetrate into many of the subcellular organelles. Especially, immunoelectron microscopic detection of intramitochondrial substances is the most difficult one, and the antibodies must penetrate through the double layered limiting membranes, unless ultrathin frozen sections are employed. From our experience with the preembedding method, satisfactory penetration through such mitochondrial walls has been obtained only by using antibodies smaller than Fab'. Indeed, we have recently succeeded in demonstrating the localization of cytochrome P450, adrenodoxin and adrenodoxin reductase in the bovine and rat adrenal cortical cells (3, 6, 7).

The outline of the staining procedure to fulfill such conditions mentioned above is as follows (11, 15, 16);

- 1) Fixation of the tissue
- 2) Washing in 0.01 M phosphate buffered saline (PBS) at pH 7.6 containing sucrose 10% up to 20%.
- 3) Freezing tissue
- 4) Cutting 4-6 μ m frozen sections by cryostat
- 5) Washing in PBS
- 6) Incubation with antibodies and peroxidase-labeled antibodies (23) 6 hr to overnight each
 - Direct method
 - Indirect method
- 7) Washing in PBS
- 8) Fixation with 2% glutaraldehyde in 0.1 M phosphate buffer at pH 7.6 for 20 min
- 9) Washing in PBS
- 10) Pre-incubation with 3,3'-diaminobenzidine-4HCl (DAB, Dotite) (20 mg/100 ml Tris HCl pH 7.6) for 30 min to 1 hr
- 11) Incubation with DAB (20 mg/100 ml Tris HCl) containing 0.005% H_2O_2 for 2 to 5 min (2)
- 12) Washing in PBS

- 13) Postfixation with 2% osmic acid in 1.0 M phosphate buffer at pH 7.6 for 1 hr
- 14) Washing in PBS
- 15) Dehydration in graded alcohol
- 16) Embedding in Epon by inverted gelatin capsule method
- 17) Ultrathin sections
- 18) Observations by JEOL 100C electron microscope

In order to obtain accurate intracellular localization of antigens, most careful attention must be paid to the fixation and the preparation of appropriate antibodies, highly specific and with proper size.

Choice of fixatives: The most effective fixative to immobilize the antigens to be localized must be selected according to the chemical nature of the antigens. In general, we have been using Zamboni's solution (26) for peptide hormones and using paraformaldehyde or PLP (periodate lysin paraformaldehyde) solution (5) for glycoprotein hormones, proteins or enzymes. For the experimental animals, we usually perfuse the animals from the left ventricle of the heart with cold PBS followed by the proper and cold fixative. Then, the excised organ or tissue blocks are fixed in the same fixative for several hr to overnight at 4°C with constant agitation. For human materials, the tissue is cut into small pieces of 2–3 mm in diameter in the fixative and fixed further at 4°C with constant agitation usually overnight. These fixatives have enabled us to obtain satisfactory preservations of both ultrastructure and the antigenicity.

Appropriate antibodies: Needless to say, the antibodies must be highly specific. In order to obtain perfect contact of antibodies with the intracellular antigens, the suitable size of antibodies must be carefully selected. For instance, intramitochondrial antigens can only be localized by the direct method with labeled antibodies smaller than Fab'. The appropriate combination of the staining method

TABLE 1. *Combination of antibodies for proper localization by pre-embedding peroxidase-labeled antibody method*

	Antibodies	Localization
1. Direct	Fab'–HRPO	Mitochondria RER
2. Direct	F(ab') ₂ –HRPO	RER Golgi saccules Cytosol
3. Indirect	Fab'——— 1st Ab Fab'–HRPO–2nd Ab	RER Golgi saccules Secretory granules
4. Indirect	F(ab') ₂ ——— 1st Ab F(ab') ₂ –HRPO–2nd Ab	RER Golgi saccules Secretory granules

Direct: Direct method Indirect: Indirect method 1st Ab: First antibody
 2nd Ab: Second antibody conjugated with HRPO HRPO: Horseradish peroxidase
 Fab': Fab' fragment of IgG RER: Cisternae of rough endoplasmic reticula

and the size of the antibodies for localization of antigens at varying intracellular sites are summarized in the Table 1.

In the field of endocrinology, the localization of antigens is anticipated in the various organelles such as perinuclear spaces, cisternae of rough endoplasmic reticula, Golgi saccules, secretory granules, and mitochondria, therefore the preembedding method with frozen sections is so far the most practical one.

2. APPLICATIONS OF ENDOCRINOLOGY

(A) *Prolactin in the rat pituitary glands*

As we have already reported (21), we have been studying the changes in ultrastructural localization of prolactin (PRL) in the rat anterior pituitary glands (22).

When the rats were sacrificed, they were perfused with Zamboni's solution and the excised pituitary glands were further fixed in Zamboni's solution overnight at 4°C with constant agitation. The previously described method of immunoelectron microscopy was performed by the indirect method first by applying anti-rat PRL anti-serum (1:100 PBS) and subsequent anti-rabbit IgG antibody IgG F(ab')₂ conjugated with horseradish peroxidase (conjugated antibody diluted 1:40 PBS) (24). Each antibody was incubated for 8 hr to overnight. The ultrastructural localization of PRL was satisfactorily performed by Zamboni's fixation and the indirect method. Antigenicity of PRL and ultrastructure were well preserved.

In the normal rats, PRL in the anterior pituitary glands was localized ultrastructurally in the perinuclear spaces, cisternae of rough endoplasmic reticula (RER), Golgi saccules and secretory granules (Figs. 1-4). At the periphery of the PRL cells, exocytosis of PRL positive secretory granules was seen (Fig. 5). Sometimes, RER were lamellated and contained PRL in the cisternae. These findings may support the proposed secretory pathway of PRL by Farquar (1), that is, RER → Golgi saccules → secretory granules → extracellular spaces.

(B) *Cytochrome P450, adrenodoxin (Ad) and Ad reductase (AdR) in the bovine and rat adrenal cortex*

The other immunoelectron microscopic approach to endocrinology is to localize the enzymes which are related to the production of hormones. We have been studying the enzyme groups of the adrenocortical cells which are related to steroidogenesis. These enzyme groups are mitochondrial cytochrome P450, Ad and AdR. The functions of these enzyme groups is to catalyze the conversion of deoxycorticosterone (DOC) to corticosterone. These enzyme groups are known to be localized biochemically in the mitochondria. By pre-embedding immunoelectron microscopy, we have demonstrated cytochrome P450, Ad and AdR in the bovine adrenocortical mitochondria and AdR in the rat adrenocortical mitochondria (Fig. 6) (3, 6, 7). As referred to earlier, the immunoelectron microscopic localization of the intramitochondrial substances can be done only by the direct method using IgG fragments smaller than Fab'. In the normal bovine and rat adrenal cortices, these enzyme groups were present in the zona fasciculata and zona reticularis. Immunoelectron microscopically, they were localized on the inner membrane of the mitochondria. We have also studied the changes of AdR in the rat adrenal cortices with experimental conditions such as hypophysectomy and ACTH administration. By hypophysectomy, the adrenocortical cells appeared to be degenerated and mitochondrial

FIG. 6. *Adrenodoxin reductase (AdR) in the zona fasciculata cells of normal rat adrenal cortex.* By pre-embedding immunoelectron microscopy, direct method by using anti-AdR IgGFab' conjugated with horseradish peroxidase, AdR was localized only in the mitochondria (arrows). $\times 8,800$

staining of AdR was decreased. When these hypophysectomized rats were stimulated with ACTH for 1 week, the hypertrophic adrenocortical cells contained many enlarged mitochondria with AdR on the inner membrane. These findings may suggest that the adrenocortical function is closely related to the status of mitochondrial steroidogenic enzymes (3).

(C) *Glutathione peroxidase (GSH-PO) in the rat adrenal cortex*

The other enzyme which has been studied in the adrenal cortex is GSH-PO. The GSH-PO catalyzes the reduction of lipid peroxides. This enzyme has been studied most extensively in the liver and erythrocytes. The immunohistochemical localization of GSH-PO has been reported by Yoshimura *et al.* (25) in our laboratory to be present in the hepatocytes. In the adrenal cortex, the enzyme is present mainly in the cells of the zona fasciculata and zona reticularis. The experimental conditions such as hypophysectomy and ACTH administration were used to clarify the role and significance of GSH-PO in the adrenocortical lipid and steroid meta-

FIGS. 1-5. *Prolactin (PRL) cells in the normal anterior pituitary gland.* By pre-embedding immunoelectron microscopic staining, PRL was localized in Golgi saccules (G), and secretory granules (SG) (arrows) (Fig. 1), in peri-nuclear spaces (PNS) (arrow) (Fig. 2), in Golgi saccules (G) and cisternae of rough endoplasmic reticula (RER) (arrow) (Fig. 3), in cisternae of RER (arrows) (Fig. 4) and in exocytotic secretory granules (arrows) (Fig. 5). Fig. 1, $\times 15,000$; Fig. 2, $\times 31,000$; Fig. 3, $\times 16,000$; Fig. 4, $\times 25,000$; Fig. 5, $\times 24,000$

bolism. When the animals were hypophysectomized, the light microscopic staining of GSH-PO was intensified in the atrophic and degenerated cortical zone which accumulated cholesterol esters. By pre-embedding immunoelectron microscopy, GSH-PO was increased in cytosol around the accumulated lipid globules. From these findings, it might well be speculated that GSH-PO could be related to the reduction of hydroperoxides of unused cholesterol esters (8, 9). The functional role of GSH-PO in the steroidogenic process is being further investigated.

As was discussed in this report, the pre-embedding immunoelectron microscopy by Nakane's peroxidase-labeled antibody method might be the most suitable method available at the present time to study the intracellular localization of the bioactive substances in the various organelles. We have demonstrated here that the pre-embedding method is capable of localizing peptide hormone in the cisternae of RER, perinuclear spaces, Golgi saccules and secretory granules and localizing adrenocortical enzymes in the mitochondria or cytosol. In the field of endocrinology, the localization of hormones as well as enzymes which are related to hormone production will prevail as valuable tools to study the functional status of the endocrine cells.

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