AN APPLICATION OF CRYOFIXATION TO IMMUNOCYTOLCHEMISTRY*

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An applicable method of cryofixation to immunocytochemistry was examined. Fresh tissue blocks of rat pancreas and parotid gland were quickly frozen by the metal contact method using liquid helium and freeze-substituted with one of the following media kept at -80°C for 36 hr; pure acetone, 0.1% glutaraldehyde (anhydrous) in acetone, approximately 0.2% paraformaldehyde in acetone, and 10% acrolein in acetone. After freeze-substitution fixation, tissue blocks were embedded in Araldite mixture. Thin sections mounted on nickel grids were processed for immunocytochemical localization of amylase according to the multiple-step protocol of the protein A-gold immunostaining method by Bendayan and Duhr (4). They were then postfixed with 2.5% glutaraldehyde in PBS and stained with uranyl acetate and lead citrate for electron microscopy. Good results were obtained from the materials substituted with glutaraldehyde or paraformaldehyde in acetone. The ultrastructural features of the cells were preserved well, similar to those in the materials substituted with OsO4 in acetone except for negative images of the membranous structures. Secretory granules, condensing vacuoles, and Golgi cisterns were labeled well with immunogold. Labeling density was much higher in the present materials than in those processed by conventional chemical fixation, and the intensity of labeling increased in proportion to increasing electron density of the materials contained within individual subcellular compartments. These results indicate that an application of the cryofixation method is useful for improving resolution and specificity in immunocytochemical postembedding staining.

Since the protein A-gold technique has been introduced in postembedding immunocytochemistry for ultrastructural localization of cellular antigenic sites (20), the technique has been widely utilized as a simple and reliable method for postembedding immunostaining in various biological systems, and improved procedures for high-resolution labeling and enhancement of immunoreactivity have developed in recent years (1–4, 6, 19, 20). However, in all of these studies, the tissue blocks were initially processed by conventional chemical fixation. This may cause artificial alterations of

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the general cellular fine structure. Recent progress in cryofixation, rapid freezing followed by freeze-substitution fixation, has made it possible to preserve well the ultrastructural features of cells and tissues (5, 8-10, 13, 18, 12, 22) as well as the chemical components contained within the subcellular compartments (11, 14, 15, 17). Thus, an application of the cryofixation method must be useful for analyzing immunocytochemical localization of antigenic sites in cells and tissues preserved more likely in their living state, although only a few studies using this approach have been reported (7, 16). The present study deals with an experimentation to examine an optimal substitution media for immunocytochemistry.

MATERIALS AND METHODS

Materials

Pancreatic and parotid acinar cells of male adult Wistar rats were used in this study. Fresh tissue blocks were excised under Nembutal anesthesia, and quenched by smashing against the polished surface of a copper block precooled with liquid helium within 30 sec after excision. The quick freezing device used in this study was a modified model of that designed by Heuser et al. (8). Frozen samples were transferred into liquid nitrogen and stored there until use.

Freeze-substitution and embedding

Frozen samples were freeze-substituted in a vial containing one of the following media kept at −80°C; pure acetone, 0.1% glutaraldehyde (anhydrous) in acetone, approximately 0.2% paraformaldehyde in acetone, and 10% acrolein in acetone. After freeze-substitution for 36 hr, the temperature was then increased to −20°C for 2 hr, to 4°C for 2 hr, and brought to room temperature. The tissue blocks were washed in 3 changes of absolute acetone (5 min each), passed through propylene oxide, and embedded in Araldite 6005 (Ciba Geigy Ltd., Basel, Switzerland) which was polymerized at 50°C.

Preparation of anti-amylase antisera

Purified amylases were obtained from rat parotid gland and pancreas, and antisera were prepared by the same method as in the previous study of Yamashita (23).

Immunocytochemical staining

Thin sections mounted on nickel grids were incubated in 1% bovine serum albumin (BSA) dissolved in 0.01 M phosphate buffered saline (PBS) for 30 min at room temperature, washed with a jet of PBS, treated overnight with anti-amylase antibodies diluted (1:250 for pancreas and 1:500 for parotid gland) with 1% BSA/PBS kept at 4°C, washed with 10 changes of PBS 3 min each, and treated with protein A-colloidal gold (Janssen Life Science Products, Beerse, Belgium) diluted 1:10 with 1% BSA/PBS for 60 min at room temperature. This was followed by incubation in rabbit anti-protein A diluted 1:200 in 1% BSA/PBS for 30 min, rinsing with 10 changes of PBS 3 min each, and then treatment with 1:10 dilution of protein A-colloidal gold in 1% BSA/PBS for 45 min. After washing with PBS in 500 ml beaker for 30 min, thin

* Paraformaldehyde acetone solution was prepared as follows; vaporized paraformaldehyde which was produced by heating powdered paraformaldehyde in flask with a gas burner was bubbled in acetone. Vaporization ceased immediately before unsolved precipitates of paraformaldehyde appeared in acetone. Concentration of paraformaldehyde in acetone was evaluated to be approximately 0.2% by measuring the weight of the flask containing acetone before and after bubbling.
sections were post-fixed with 2.5% glutaraldehyde in PBS, washed with distilled water for 15 min, and then stained with uranyl acetate and lead citrate for electron microscopy. For the immunological control, 1) the antisera were absorbed with pancreatic amylase and with parotid gland amylase respectively, and the resulting supernatants were used for immunoreaction, 2) an irrelevant antibody, rabbit anti-human albumin antibody (Cosma Bio Co. Ltd., Tokyo, Japan) was used in place of the specific antibodies, and 3) the specific antibodies were replaced by rabbit normal serum in immunostaining. Concerning the control for the effect of the cryofixation, the same samples were processed by conventional chemical fixation, and embedded in an Epon-Araldite mixture which was polymerized at 60°C. Their thin sections were immunostained with the same method, and labeling density in both sections was compared.

RESULTS

The cryofixation employed here preserved well the ultrastructural features of the glandular cells. They were characterized with well-developed parallel arrays of flattened cisterns of the granular endoplasmic reticulum, widely-spread and continuous Golgi stack with fenestrae, and mitochondria with the dense matrix (Fig. 1). Individual cisterns of the endoplasmic reticulum were of uniform width and contained a flocculent material which appeared denser than the cytoplasmic matrix. Stacked cisterns of the Golgi apparatus and the associated small vesicles also contained a moderately dense material. Its density varied from cistern to cistern, and an increasing gradient of electron density from the cis-side, through the trans-side, to condensing vacuoles was recognized. Zymogen granules of the pancreatic acinar cell contained a dense homogeneous content, whereas those in the parotid acinar cell appeared to contain a heterogenous interior in the cryofixed materials. On the contrary, irregularly distended cisterns of the granular endoplasmic reticulum as well as fragmented Golgi stack were observed in the materials processed by conventional chemical fixation (Fig. 2), and they appeared empty.

Freeze-substitution with aldehyde-containing acetone was effective to show moderately high image contrast of the cellular structures, although the membranous components appeared as negative images (Fig. 3). Acrolein in acetone was not very good for fixation, because the cytoplasmic matrix appeared finely granulated and rough in texture. Pure acetone showed only a poor fixation effect.

Zymogen granules in either pancreatic or parotid acinar cells processed with the present cryofixation were more intensely labeled with immunogold than those in the materials processed by conventional chemical fixation (Figs. 2, 3). Gold particles were distributed homogeneously over dense homogeneous content of secretory granules in the pancreatic acinar cells, while in the parotid acinar cells they were heterogeneously distributed in proportion to heterogeneous pattern of electron density of the granular matrix (Figs. 4, 5). That is, the higher the electron density of the matrix area was, the more intensely labeled with immunogold. The trans-side of the Golgi stack was moderately labeled with immunogold (Figs. 3, 5), and the cis-side of the Golgi apparatus as well as the granular endoplasmic reticulum were only faintly stained by the present method. Non-specific background labeling over the cytoplasmic matrix and/or the intercellular space was virtually absent.
Fig. 1. The Golgi area in the cytoplasm of the rat pancreatic acinar cell processed by rapid freezing followed by freeze-substitution with 0.1% glutaraldehyde in acetone. Note parallel arrays of flattened cisternae of the granular endoplasmic reticulum and a continuous, multiple stacked cisternae of the Golgi apparatus. Discontinuity of the stack represents a fenestra for traffic of the small vesicles (arrow). cv: condensing vacuole, m: mitochondria, z: zymogen granule. ×19,000

Fig. 2. Rat pancreatic acinar cell processed by conventional chemical fixation and stained with anti-rat pancreatic amylase antiserum. The ultrastructural features of the cells are not very well preserved in comparison with those shown in Fig. 1. Individual zymogen granules, condensing vacuoles as well as luminal contents are moderately labeled with protein A-gold complex. Inset; immunogold labeling of secretory granule in higher magnification. ×11,000 Inset. ×22,000

Fig. 3. A cross-sectional area of the Golgi field of rapid-frozen, substitution fixed pancreatic acinar cell. Labeling density on zymogen granules as well as the Golgi cisternae is much higher than that shown in Fig. 2. ×21,000
DISCUSSION

Among substitution media examined, aldehyde-containing acetone was the most effective for preservation of the fine structural features of the pancreatic and parotid acinar cells, despite omitting osmium tetroxide from the media. Characteristic features of these cells, such as flocculent material in the RER cisterns and a gradient of increasing electron density of materials contained within the Golgi cisterns and condensing vacuoles, clearly indicate that the present cryofixation retained well not only the cell fine structure but also macromolecular substances which are possibly synthesized and processed within these organelles. The facts that labeling density was much higher in the cryofixed materials than in those processed by conventional chemical fixation, and labeling intensity increased in proportion to higher electron density of the substance contained represent an excellent preservation of antigenicity of amylase in

The positive reaction was not recognized in the control experiments, using absorbed antibodies, irrelevant antibody and normal rabbit serum in place of the specific antibodies.

Fig. 4. Control preparations of the parotid acinar cell in which the antiserum was replaced by normal serum. The tissue block was freeze-substituted with approximately 0.2% paraformaldehyde in acetone. Note that the contents of zymogen granules (z) are heterogeneous in appearance. cv: condensing vacuole, G: Golgi apparatus, l: lumen. × 20,000
the cells processed by the present cryotechnique.

In the parotid acinar cells processed by the present cryofixation, secretory granule content appeared heterogeneously. It is known that secretory granules of rat parotid acinar cells are seromucous in nature and have a dense homogeneous content in matured stage, but they may exhibit various appearances of heterogeneity due to the fixation (24). The present observation is considered to reflect the granule content in the living cells, because with rapid freezing it is possible to fix physically the cellular components within a few milliseconds and minimize to production of artificial alterations of the cell structure. Similar findings were also observed in other seromucous cells processed by the cryofixation (12, 14). The disadvantage of the present cryofixation is negative images of the membranous components of cells. This makes it difficult to define localization of labeling on obliquely- or tangentially-sectioned areas of small vesicles and/or flattened cisternae because of unclear contours. This must be improved by freeze-substitution with osmium-containing media and pretreatment of thin sections with oxidizing reagents such as hydrogen peroxide or sodium metaperiodate according to Bendayan and Zollinger (3).

In any event, it is evident that an application of the cryofixation to indirect immunostaining technique is useful for enhancement of immunoreactivity and high-

Fig. 5. Apical cytoplasm of the same parotid acinar cell as in Fig. 4, after immunostaining. Labeling density on zymogen granules is high in proportion to electron density of granular contents. G: Golgi apparatus. \( \times 20,000 \)
resolution localization of antigenic sites in cells and tissues preserved more likely in their living state.

REFERENCES


