Effect of Fixation with Reduced Osmium Tetroxide upon the Antigenicity of Liver Catalase and Erythrocyte Esterase D

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Effect of reduced OsO₄ fixation on the antigenicity of rat liver catalase and erythrocyte esterase D was investigated. After fixation with mixture of 4% paraformaldehyde and 0.2% glutaraldehyde, rat liver and erythrocytes were post-fixed with reduced osmium (0.1% to 1.0%) for 1 hr. Some erythrocytes were fixed with 0.5% reduced osmium for various times (10 min to 60 min). The antigenicity of both enzymes was expressed as labeling density (gold particles/µm² of peroxisomes or erythrocytes). Fixation with reduced OsO₄ greatly affected the antigenicity of both enzymes. Approximately 80% of catalase antigenicity was lost at 0.4% reduced OsO₄, whereas about 80% of esterase D antigenicity was lost at 0.2%. Positive contrast of membrane structures clearly appeared at 0.6% OsO₄. About 80% loss of the esterase D antigenicity was observed at 10 min after fixation with 0.5% reduced OsO₄. Afterwards, the antigenicity decreased very slowly. The data indicates that at the concentration of reduced OsO₄ which stains the membrane structures positive contrast, 80% of the antigenicity is lost in the cases of catalase and esterase D.

Key words: Post-embedding immunocytochemistry, Reduced OsO₄, Antigenicity, Catalase, Esterase D

I. Introduction

Post-embedding immunocytochemistry is now used widely in the field of cell biology and medicine. Electron microscopic image of membranes structures in the post-embedding immunocytochemistry is of negative contrast as observed after negative staining of cell fractions with phosphotungstic acid or uranyl acetate. This is due to omission of post-fixation with OsO₄. It is known that OsO₄ reacts with lipid molecules of membrane structures so that the membrane structures exhibit positive contrast [4]. If the post-osmification is omitted, all of the membrane structures are not stained and consequently exhibit negative contrast after usual contrasting with uranyl acetate and lead citrate. In the post-embedding methods using acryl resins as a embedding medium, OsO₄ is not used in general, because the strong oxidative activity of this chemical crucially disturbs polymerization of the resins.

To obtain a positive image of the membrane structure in the post-embedding immunocytochemistry, fixation of tissue has been improved by several groups. Berryman and coworkers used tannic acid for post-fixation [2]. Nanci et al. fixed tissues with potassium ferrocyanide-reduced OsO₄, followed by embedding in Lowicryl K4M [7]. Fixation with reduced osmium was first introduced by Karnovsky [5] who showed that reduced osmium enhances the contrast of the membrane structures. Furthermore, if a low concentration of potassium ferrocyanide is used for reduction of OsO₄, surface coat of the cell membrane is also stained [3]. Tamaki and Yamashina have also applied reduced osmium to post-embedding immunocytochemistry of Golgi apparatus [11]. Another approach to obtain the contrast of membrane structures on ultrathin sections of Lowicryl K4M-embedded materials was made by Roth and coworkers [10], who applied the staining method of uranyl acetate-methyl cellulose adsorption that was used in ultracytosections. They did not use OsO₄ fixation.

In studies using OsO₄, the membrane structures are shown clearly as a positive contrast. However, the effect of fixation with reduced OsO₄ upon antigenic activity has not yet been clearly documented. In this paper, the effect of reduced osmium on the antigenicity of catalase and erythrocyte esterase D was investigated by quantitative immunocytochemistry. Catalase is contained almost homogeneously in every peroxisome of normal rat liver. Erythrocyte esterase D also distributes evenly in the cytoplasmic matrix of every erythrocyte. Therefore, there

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is no problem in statistical estimation of immunogold labeling for these enzymes. The results showed that the antigenicity of both the enzymes, expressed by labeling density, was lost seriously after post-fixation with reduced osmium and was decreased 1/5 of control at concentration of 0.6% OsO₄, at which the membrane structures had a clear positive contrast.

II. Materials and Methods

Antibodies

Rabbit polyclonal anti-rat liver catalase was described previously [13]. Anti-human erythrocyte esterase D was raised in Japanese white rabbits immunized with purified esterase D [9]. Monospecificity was tested by immunoblot analysis using rat erythrocyte extract. Both antibodies developed a single band with molecular mass of 60 kDa (catalase) and 33 kDa (esterase D), respectively.

Pre-fixation

Male Wistar rats weighing 200–250 g were used. Liver was fixed by perfusion through a portal vein for 5 min. The fixative consisted of 4% paraformaldehyde, 0.2% glutaraldehyde and 0.01% CaCl₂ in 0.15 M cacodylate buffer (pH 7.4). Liver tissue was sliced into sections 100 µm thick and the resulting slices were cut into about 1 mm squares. Erythrocytes were isolated from 10 ml of rat blood in the presence of heparin, washed twice with PBS by centrifugation and suspended in 10 ml PBS. One ml of the erythrocyte suspension was mixed with the same volume of fixative consisting of 8% paraformaldehyde, 0.4% glutaraldehyde, 0.02% CaCl₂ and 0.3 M cacodylate buffer (pH 7.4), and allowed to stand for 30 min. After fixation, cells were washed thoroughly with PBS.

Post-fixation

One percent reduced osmium was prepared as follows. Potassium ferrocyanide (7.5 mg) was dissolved in 0.25 ml of distilled water. This solution was immediately mixed with 0.25 ml of 2% osmium tetroxide aqueous solution. The amount of potassium ferrocyanide was changed in direct proportion to the concentration of osmium tetroxide. Pre-fixed liver tissue slices and some erythrocytes were post-fixed in various concentrations (0.1% to 1.0%) of reduced osmium for 1 hr and washed three times with PBS. Other pre-fixed erythrocytes were post-fixed with 0.5% of reduced osmium at various lengths (10 min to 60 min). After post-fixation, erythrocytes were centrifuged down in agarose warmed at 50°C and the resulting pellets were cut into small blocks. Liver tissue slices and erythrocyte blocks were then dehydrated in graded dimethylformamide and embedded in LRWhite at −20°C. Polymerization of the resin was performed under UV light at −20°C.

Immunocytochemical staining

Thin sections mounted on uncoated nickel grids were incubated with 0.5% bovine serum albumin for 5 min, followed by overnight incubation with specific antibodies at 4°C. After being washed, sections were incubated with protein A-gold probe (15 nm gold) for 30 min. Sections were then stained with uranyl acetate and lead citrate and observed in a Hitachi H600 electron microscope.

Quantification of labeling density

Ten Electron micrographs were taken at a magnification of 15,000 for each group and enlarged to a final magnification of 40,000. The areas of peroxisomes and the erythrocyte cytoplasm were measured by semi-computing system [12], respectively. Next, gold particles in the respective area were counted with a colony counter. Labeling density was expressed as gold particles per µm².

III. Results

Effect of the concentration of reduced osmium on image of membrane structures

The effect of reduced osmium on staining of membrane was observed in liver cells. As shown in Figure 1, the membrane structures became stained positively at a concentration of more than 0.4% of osmium. At 0.1 to 0.2%, membranes were so weakly stained with osmium that total image of the membrane structures were rather unclear comparing with osmium-unfixed material (Fig. 1-b, c). At 0.6% the membranes were clearly contrasted (Fig. 1e) and were similar to those seen in routine electron microscopy.

Effect of the concentration of reduced osmium upon the antigenicity revealed by quantitative immunoelectron microscopy

Labeling density for catalase gradually decreased as the concentration of reduced osmium increased (Fig. 2). At 0.4% and 0.6% approximately 80% of antigenicity was lost. By esterase D, at 0.1% of reduced osmium 50% of the antigenicity was lost (Fig. 3). The majority of decrease in the labeling density rapidly occurred around 0.2% of reduced osmium, afterwards the antigenicity decreased gradually (Fig. 3). The loss of esterase D antigenicity occurred already 10 min after fixation with 0.5% reduced osmium (Fig. 4), afterwards it was lost very gradually.

IV. Discussion

In present study, it was shown that the positive image of membranes appeared after fixation with more than 0.4% of reduced osmium. At 0.2% of OsO₄ membrane structures were rather unclear compared with those of osmium-unfixed material, because they were neither negative nor positive images. Therefore, in order to get clear positive profiles of membrane structures, more than 0.4% of reduced osmium should be used. However, the
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Fig. 1. Effect of reduced osmium on membrane structures and immunolabeling. A: a liver cell fixed only with paraformaldehyde-glutaraldehyde mixture. Membrane structures are negative image and peroxisomes are heavily labeled with gold particles. B: a liver cell fixed with 0.1% OsO₄. Membrane structures are indistinct. C: 0.2% OsO₄-fixed tissue. D: 0.4% OsO₄-fixed tissue. Membranes are positively contrasted. E: 0.6% OsO₄-fixed tissue. Membrane structures are clearly contrasted but labeling for catalase considerably decreases comparing with A. × 28,000.

Fig. 2. Effect of reduced OsO₄ concentration on the antigenicity of rat liver catalase. Relative labeling density is ratio to labeling density obtained from OsO₄-unfixed material.

Fig. 3. Effect of reduced OsO₄-concentration on the antigenicity of rat erythrocyte esterase D.

Fig. 4. Effect of fixation time with reduced OsO₄ on the antigenicity of rat erythrocyte esterase D.

Present study clearly showed that 75% of the antigenicity of catalase expressed by labeling density was lost at this concentration. Similarly, the antigenicity of erythrocyte esterase D was reduced to about 20% of osmium-unfixed material. Thus, fixation with osmium has a strong harmful effect on the antigenicity of both catalase and esterase D. Perhaps, this deleterious effect might be true for many other protein antigens. Since the loss of the antigenicity depends upon the concentration of reduced osmium, osmium directly involves in destruction or change of the epitopes.

Osmium tetroxide not only reacts with membrane lipids but also can cross-link proteins in vitro [3]. However, it is known that the effects of osmium are often destructive. For example, OsO₄ treatment completely destroys actin filaments within minutes [6]. Furthermore, it was shown that OsO₄ cleaves some peptide bonds in proteins [4]. Together with this evidence, catalase and esterase D examined in the present study were also cleaved by reduced OsO₄, so that their antigenicity was remarkably
lowered. Such cleavage can be blocked in vitro by tertiary amines [1, 4]. This suggests that the loss of the antigenicity by osmium fixation could be suppressed by use of tertiary amines. Marked decrease of antigenicity by osmium fixation means a possibility that originally small amount of antigens cannot be detected after fixation. Although our present data might not be applicable to all protein antigens, the following points should be considered when reduced osmium is used for fixation; 1) antigens to be detected are highly concentrated, 2) the antigens is resistant to osmium, and 3) about 80% of antigenicity is possibly lost after fixation with 1% OsO₄.

In conclusion, reduced osmium is strongly harmful for protein antigens. At the concentration used in routine electron microscopy, more than 80% of antigenicity is lost in the cases of rat liver catalase and erythrocyte esterase D. Fixation with reduced osmium should be carefully applied to post-embedding immunocytochemistry.

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VI. References


