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Antigen retrieval on epoxy sections based on tissue infiltration with a moderately increased amount of accelerator to detect immune complex deposits in glomerular tissue

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Summary. We wanted to examine the effect of antigen retrieval on epoxy sections where the tissue had been infiltrated by resin containing moderately increased amounts of accelerator. The concentration of accelerator DMP-30 (Tri(Dimethyl Amino Methyl) Phenol) was varied in the range of 0% to 4% in the infiltration step of the tissue processing. Some of the epoxy sections were fixed in osmium tetroxide, and for others this fixative was avoided. Immunogold labeling was performed on epoxy sections and LR-White sections of renal tissue with IgG-deposits, and the antibody used was anti-IgG. Antigen retrieval was performed by heating the sections in citrate buffer. The amount of immunogold labeling on retrieved sections increased according to the amount of accelerator the non-osmicated epoxy sections were based on in the infiltration steps. For the osmicated epoxy sections these differences were less pronounced. The immunogold labeling of retrieved epoxy sections was up to 70% of LR-White labeling. In addition to breaking fixation bond introduced by the chemical fixation, we believe that the antigen retrieval also breaks bonds between the epoxy resin and the embedded tissue. The combination of increased amount of accelerator in the tissue infiltration and antigen retrieval by heating the sections in citrate buffer is a good method for improving the immunolabeling of epoxy sections.

Key words: Antigen retrieval, immunocytochemistry, embedding

Introduction

Acrylic resins are usually better suited for immunolabeling than conventional epoxy resin (Newman, 1989; Brorson and Skjørten, 1996a). Especially large proteins have been difficult to immunolabel on epoxy sections, while small antigens are easier to immunolabel on these sections (Ottersen, 1989; Brorson and Skjørten, 1996a). The reason why acrylic resins are often preferred in immunoelectron microscopical procedures is the following: epoxies are able to form covalent bonds with biological materials, particularly with proteins. Co-polymerization of epoxies with embedded tissues occurs, while polymerized acrylic resins surround embedded tissues without binding to them. Accordingly, during cleavage the behaviour of epoxies and acrylics is different from each other. Without co-polymerization (acrylics), the surface of cleavage tends to follow the areas of least resistance, e.g., the interfaces between resin and proteins. In epoxy- embedded material, however, the resistance in these interfaces is not significantly less than in proteins. When cutting epoxy-embedded tissue, the surface of cleavage has greater tendency to divide the proteins (Kellenberger et al., 1987). According to this theory of Kellenberger, the surface of conventional epoxy sections is smoother than LR-White sections. Epoxy sections usually give better preservation of ultrastructural details than acrylic sections, they are more stable when exposed to the electron beam, and epoxy resin blocks are also easier to trim and cut. Several methods have been applied to enhance the immunolabeling for epoxy sections (Brorson, 1998) including etching with chemicals (e.g. sodium ethoxide) (Mar and Wight, 1988; Brorson and Skjørten, 1995; 1996b; Stirling and Graffs, 1995; Brorson, 1997a), and by large increases in the amount of accelerator in the infiltration and embedding steps when processing the tissue (Brorson and Skjørten, 1996c; Brorson et al., 1997). The high-accelerator method is especially useful for labeling large proteins (Brorson and Skjørten, 1996c), and this method has been used for routine immunoelectron microscopy on renal biopsies in order to detect immune complex deposits (Brorson et al., 1997). The mechanism behind the increased immuno-

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labeling on high-accelerator epoxy sections is thought to be reduced co-polymerization between the tissue and the resin. But the high-accelerator method for routine immunolabeling of renal biopsies also has its shortcomings due to reduced infiltration properties, reduced contrast of the specimens, and to the phenomenon that renal biopsies with high-accelerator cannot be processed in an ultraprocessor together with other tissues. Therefore, it would be desirable to have a method reducing these problems. Since several researchers have had good experiences with antigen retrieval by heating the sections in citrate solution (Boon and Kok, 1994; Wilson et al., 1996), it is reasonable to examine if a combination of such heating of the sections and a moderate increase in the amount of accelerator in the infiltration of the tissue gives improved immunolabeling.

Materials and methods

Antibody: rabbit anti-swine-IgG (1:200) (Nordic, Tilburg, Netherlands).

Colloidal gold probe: Goat anti-rabbit IgG, Auroprobe EM Gar G15 (Amersham, Little Chalfont, Bucks, England).

Substrates: Kidney tissue was obtained from Yorkshire swine (from the slaughter house 30 min after death). The swine kidney tissue included glomerular immune complex deposits with immunoreactivity against IgG.

Procedures

The tissue was cut into small pieces (<1 mm), fixed in 4% paraformaldehyde and 1% glutaraldehyde in phosphate buffer (pH 7.3) at 4 °C overnight, and washed for 2 hr with 0.2M cacodylate buffer (pH 7.3). Some of the specimens were postfixed in 1% osmium tetroxide in 0.2M cacodylate buffer for 1 h or 2 h prior to embedding in epoxy resin.

Embedding in LR-White (London Resin Company Ltd, Basingstoke, Hampshire RG22 5AS, UK):

1) 70% ethanol for 20 min.
2) 2 x 96% ethanol for 20 min.
3) Mixture of LR-White and 96% ethanol (1:1) for 2 hr.
4) Pure LR-White for 5 hr.
5) Embedding: In gelatine size 1 capsules.
6) Polymerization: 56 °C for about 40 hr.

Embedding in epoxy resin: the tissue was dehydrated, infiltrated and embedded according to the following procedure:

1) 70% ethanol for 20 min.
2) 96% ethanol for 20 min.
3) 3 x 100% ethanol for 20 min each.
4) 3 x propylene oxide for 20 min each.
5) Mixture of 50% LX-112 (Epoxy resin from Ladd Inc., Burlington, Vermont, USA) and 50% propylene oxide for 6 h
6) Pure LX-112 mixture for 10 h.
7) Embedding: pure LX-112 mixture.

The concentration of accelerator (DMP-30) was varied in the infiltration steps. We use the following nomenclature to describe the concentrations of accelerator in the infiltration and embedding steps (Brorson and Skjørten, 1996c). The term *"[x.y.z]-embedding"* will describe an infiltration-embedding procedure with x% accelerator in the first infiltration step (where epoxy resin and propylene oxide are mixed), y% accelerator in the second infiltration step (with pure epoxy resin) and z% accelerator in the final embedding step (The concentrations of accelerator refer to the LX-mixture before being eventually mixed with propylene oxide). Similarly, *[x.y.z]-sections* are cut from specimen blocks that are produced by [x.y.z]-embedding. The following 4 types of epoxy embeddings were used: [0.0.2], [2.2.2], [3.2.2], and [4.2.2], which means that the concentration of accelerator was varied in the range of 0% to 4 % in the first infiltration step (the resin/propylene oxide step). The specimens were embedded in gelatine capsules and polymerized at 56 °C for 3 days.

Cutting

Thin sections with gold interference color of LR-White, and epoxy-embedded tissue were cut with a diamond knife (Jumdi, Juniper Ultra Micro, Stockholm, Sweden) on an LKB 2088 Ultratome V. The sections were mounted on 200-mesh nickel grids.

Antigen retrieval

Some epoxy sections based on different amounts of accelerator ([0.0.2], [2.2.2], [3.2.2], and [4.2.2]) and with or without postfixing with osmium tetroxide were exposed to heating in citrate buffer (0.01M, pH 6.0) at 95 °C for 15 minutes in a PCR-machine (GeneAmp 2400, Perkin Elmer).

Immunoprocedure

Immunogold labeling was performed on grid-mounted sections floating in drops according to Brorson et al. (1994). Osmicated epoxy sections were oxidized by saturated $NaIO_4$ for 1 h at room temperature before the antibody-incubation. An incubation step with 10% BSA (diluted in PBS, pH 7.2) for 4 hours was introduced prior to immunolabeling to block non-specific labeling (Brorson, 1997b). The primary antibody, anti-IgG, was diluted in 10% BSA, and the incubation was carried out overnight at 4 °C. The secondary immunoreagent (antibodies coupled to 15 nm colloidal gold particles) was diluted in 10% BSA, and the conditions of incubation were 75 min at 22 °C. After immunolabeling, the sections were stained with 5% uranyl acetate in 30% ethanol for 20 min, and Reynolds lead citrate for 20 min.

Immunostained sections were examined and photographed in a transmission electron microscope (Jeol 1200 EX). The calibrated magnification was determined with a replica grid (2160 lines/mm cross

ruled grating replica). The sections of glomerular tissue were photographed at a display magnification of x10,000. Measurements and calculations from the photographs were performed in the following way: areas of antigen location on the sections were randomly chosen and examined for degree of labeling in terms of number of gold particles per μm^2. In each case 10 photographs from ten different locations were used for the calculation of the number of gold particles per μm^2.

The photographs were scanned into a Power Mac 6100 with Adobe Photoshop, and areas and the number of gold particles were automatically counted by the image processing program NIH-Image 1.60. The relative labeling was finally expressed relative to the immunogold labeling of the LR-White sections which was set equal to 1.00 as the reference value (a relative labeling of 1.00 is equal to 211 gold particles per μm^2).

Results

When comparing the labeling of retrieved non-osmicated epoxy sections, the immunogold labeling was more intense the more accelerator their tissue processing was based on; the relative labelings were as follows (± standard error): [0.0.2]-sections: 0.35±0.04; [2.2.2]-sections: 0.51±0.04; [3.2.2]-sections: 0.56±0.03; [4.2.2]-sections: 0.67±0.03. The labeling for IgG on retrieved [4.2.2]-sections was about 70% of the labeling on LR-White sections (Fig. 1). For osmicated epoxy sections exposed to oxidation and antigen retrieval the tendency of the results was somewhat different in the sense that there were no significant differences in the immunogold labeling on [2.2.2]-sections (rel. label.=0.35±0.03), [3.2.2]-sections (rel. label.=0.33±0.02), or [4.2.2]-sections (rel. label.=0.32±0.02). There was a clear and significant difference in immunolabeling between these three types of sections on the one hand and the equally treated osmicated [0.0.2]-sections (rel. label.=0.16±0.01) on the other. There were no significant differences in immunolabeling due to if the tissue was fixed for 1 h or 2 h in osmium tetroxide (Fig. 2). In all cases the labeling for IgG was less intense on oxidized and retrieved osmicated sections than on retrieved non-osmicated sections. The non-specific background labeling on the glomerular tissue on oxidized, retrieved osmicated epoxy sections was about 15% of the specific labeling, while the non-specific labeling was about 5% of the specific labeling on the retrieved, non-osmicated epoxy sections. The non-specific labeling on the retrieved sections did not increase significantly in the direction of increased amount of accelerator. Osmicated sections which were not exposed to antigen retrieval or oxidizing were negative for specific labeling against IgG, and non-retrieved, non-osmicated [0.0.2]-sections were almost negative (rel. label.=0.03).

The ultrastructural preservation of both osmicated and non-osmicated sections seemed good, but the cell borders were more distinct on the osmicated sections.

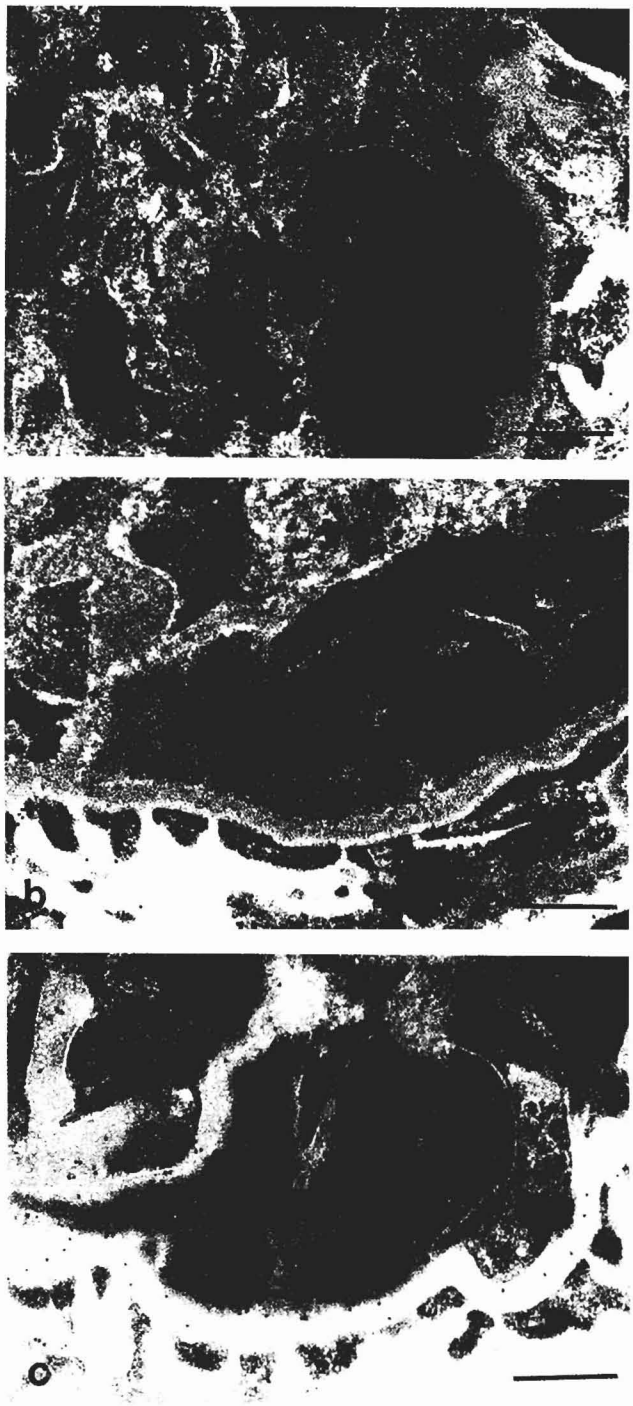

Fig. 1. The immunolabeling of immune complex deposits with anti-IgG in (a) [0.0.2]-section, (b) [4.2.2]-section, and (c) LR-White section. These sections are not osmicated, and (a) and (b) are exposed to antigen retrieval, while (c) is not retrieved. For the [0.0.2]-section the immunolabeling is distinct, but not very intense, while the labeling on the [4.2.2]-section is distinct and intense, and only about 30% less intense than for the LR-White section. x 27,300, Bar: 500 nm.

The sections which were oxidized by $NaIO_4$ were paler than other sections. The contrast of all the retrieved, non-osmicated sections was the same as that observed for conventional epoxy sections.

Discussion

Over the last years methods have been developed where sections are heated in a retrieval medium to enhance the immunolabeling of antigens for immunohistochemistry (Taylor et al., 1996). The mechanism for antigen retrieval by heating in citrate buffer is assumed to be the breakage of some of the fixation bonds introduced by the chemical fixation, and thereby the epitopes are more recognisable for the antibodies (Boon and Kok, 1994). We believe that an additional explanation is needed to understand the mechanism for antigen retrieval of epoxy sections. When embedding tissue in epoxy resin, co-polymerization occurs between side groups of proteins and the polymer chains (Causton, 1984; Kellenberger et al., 1987), and this results in fewer epitopes exposed at the surface of the epoxy sections than on the LR-White sections, especially for large proteins (Brorson and Skjørten, 1996a). We believe that the reactions between side groups of proteins and the epoxy polymer often occur with the fixative as a link. When the tissue is fixed, side groups of proteins (especially $-NH_2$ groups) are occupied by the fixative. This often occurs in a non-crosslinking way so the aldehyde molecules have one free reactive group. When the fixed tissue is embedded in epoxy resin, the epoxy chains may react with the fixative molecules attached to the protein, and thereby the protein and the epoxy resin are co-polymerized. When the epoxy sections are exposed to antigen retrieval by heating in citrate buffer, the bonds between fixative and proteins are broken. As a result of this breakage, the proteins are at least partly released from the epoxy network, and more epitopes are exposed to the immunoreagents because of a superficial deplasticizing. There are fewer bonds between the epoxy resin and the tissue when more accelerator is used in the processing (Brorson and Skjørten, 1996c). The observation of higher immunolabeling of the retrieved epoxy sections with more accelerator present shows that breakage of epoxy-protein bonds in the low accelerator epoxy sections is not enough to surpass the combined effect of reduced co-polymerization in the accelerator-increased epoxy sections and the breakage some of its relatively few epoxy-protein bonds by retrieval.

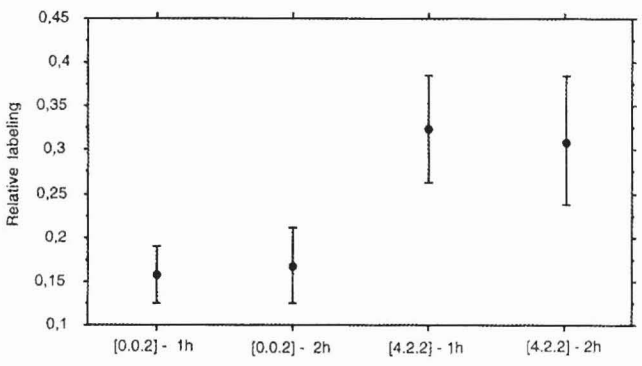

Fig. 2. The relative immunolabeling (with standard deviation error bars) for IgG on osmicated [0.0.2]- and [4.2.2]-sections after exposure to oxidizing and antigen retrieval. There are no significant differences if the osmium fixation has endured for 1h or 2h.

Osmium tetroxide is a strong fixative, not only for lipids, but also for proteins, and therefore contributes to bind the proteins more strongly in the network of proteins, fixatives and epoxy resin. When the sections are exposed to $NaIO_4$, the negative effect of osmium tetroxide on the immunolabeling is only partly reversed. We believe that this effect of osmium tetroxide smoothes out the effect of increased accelerator on the immunolabeling for [2.2.2]-sections, and [3.2.2]-sections and [4.2.2]-sections in such a way that significant differences in immunogold labeling cannot be discovered by the present method. Prolonged fixation with osmium tetroxide may cleave proteins (Baschong et al., 1984), and this effect cannot be reversed by oxidizing with $NaIO_4$. Since there were no differences in the immunolabeling if the osmium fixation lasted for 1 h or 2 h, we believe that the IgG antigens were not increasingly cleaved by osmium fixation by extending the duration of the fixation from 1 h to 2 h.

In conclusion, we have established that a moderately increased amount of accelerator in the infiltration of kidney tissue with epoxy resin gives a significant increase in the immunolabeling of IgG on immune complex deposits when the sections are heated in a citrate solution. We recommend a [4.2.2]-embedding without osmium to obtain a good immunolabeling with low background labeling and good contrast. This embedding method is compatible with processing in an automatic ultraprocessor together with most other tissues. Since this resin is less viscous than high-accelerator epoxy resin ([8.4.6]-embedding), the infiltration of most tissues will be non-problematic. Combined with antigen retrieval by heating in citrate buffer, [4.2.2]-sections give almost as high a labeling as LR-White sections, and epoxy sections are to be preferred because of good ultrastructural preservation, better sectioning qualities and better stability in the electron beam.

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