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Review

Heat-induced antigen retrieval of epoxy sections for electron microscopy

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Summary. The purpose of this manuscript is to review the literature on the use of heat-induced antigen retrieval methods to enhance the immunolabeling of epoxy sections at the electron microscopical level. The history of the development of antigen retrieval by heating formaldehyde fixed paraffin sections in a buffer solution is given in short, and how this technique has been extended to resin sections and in particular epoxy sections is explained. Theories for the mechanism of enhancement of the immunolabeling of epoxy sections by the heat-retrieval method are discussed, and it is finally speculated whether most of the mechanisms for antigen retrieval on epoxy sections in heated buffer solution are essentially the same as for conventional immunoenhancing by deplastizing and etching. The more accelerator used in the processing of the tissue the more intense the immunolabeling of the heated epoxy sections becomes. The intensity of immunolabeling of the epoxy sections increases with the temperature in the heated buffer solution, and the intensity is significantly higher at high autoclave temperatures than at 95 °C, Heat-induced antigen retrieval is also compared with other, conventional techniques for enhancing the immunolabeling of epoxy sections.

Key words: Antigen retrieval, Epoxy resin, Immunohistochemistry, Acrylic resin, Immunogold

I. Introduction

IA. Short history of antigen retrieval for light microscopy

Formaldehyde is the most commonly used fixative for tissue preparation for light microscopy. This fixative - or oligomers of them - crosslink proteins by binding to side groups of the proteins, especially amino groups. Formaldehyde protein bonds are unstable and can be hydrolysed in aqueous environment (Hayat, 1989). Over the last 10 years methods have been developed where sections are heated in a retrieval medium in a microwave oven, pressure cooker or on a cooking plate for 10-20 min to enhance the immunolabeling of antigens for routine immunohistochemistry (Boon and Kok, 1994). Antigen retrieval by heating the sections in buffer solution was first performed by Shi et al. (1991). The most commonly used medium for antigen retrieval is citrate or EDTA buffer, but other aqueous media with other salts like e.g. zinc sulphate, lead thiocyanate and aluminium chloride have also been used (Boon and Kok, 1994). The enhancement of immunolabeling by heating is explained by releasing the bonds introduced by the fixation of the tissue in formaldehyde. Some researchers also claim that EDTA and citrate release Ca^{2+} and Mg^{2+} ions from the tissue and thereby facilitate the immunodetection of the antigens (Morgan et al., 1994). Today, antigen retrieval of formaldehyde-fixed paraffin sections is a standard procedure in routine diagnostics in pathology laboratories.

IB. Some chemical qualities of epoxy resins

Tissue embedding for electron microscopy is performed by resin infiltration and polymerization. Epoxy resin and acrylic resins are the most commonly used resins. The immunolabeling is almost always performed by having small gold particles (1-15 nm) as the immunomarker, and is therefore called 'immunogold labeling'. Acrylic resins usually give higher intensity of immunogold labeling than conventional epoxy resin (Newman, 1989; Brorson and Skjørten, 1996a). The reason why acrylic resins are often preferred in immunoelectron microscopical procedures is explained by Kellenberger et al. (1987): "Epoxies are able to form covalent bonds with biological materials, particularly with proteins (the epoxy polymers may probably bind proteins with the fixation molecule as a link). Co-polymerization of epoxies with embedded tissues occurs, while polymerized acrylic resins surround embedded tissues without binding to them. Accordingly, during cleavage/sectioning the behaviour of epoxies and acrylics is different from each other. Without co-

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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 plane of sectioning has greater tendency to cleave the proteins." In this way the surface of conventional epoxy sections becomes smoother than acrylic sections.

It is well known that epoxy sections usually give better preservation of ultrastructural details than acrylic sections, that they are more stable when exposed to the electron beam, and that epoxy resin blocks are easier to polymerize, trim and cut. Several conventional methods have been applied to enhance the immunolabeling for epoxy sections (Brorson, 1998a) including etching with chemicals (e.g. sodium ethoxide) (Mar and Wight, 1988; Brorson and Skjørten, 1995, 1996b; Stirling and Graff, 1995; Brorson, 1997). Sodium ethoxide has extremely high pH, and may dissolve the ester-like bounds in the epoxy resin totally (Mar and Wight, 1988) or partly (Brorson, 2001). Other methods in this category are etching with H_2O_2 (Holm et al., 1989; Brorson et al., 2001) or sodium metaperiodate (Stirling and Graff, 1995).

Another way of increasing the intensity of immunolabeling is by increasing the amount of accelerator in the infiltration and embedding steps when processing the tissue (Brorson and Skjørten, 1996c; Brorson et al., 1997). The mechanism for increased intensity of immunolabeling is reduction of the copolymerization between the epoxy resin and the biomolecules.

IC. Antigen retrieval of ultrathin sections for electron microscopy

Antigen retrieval of epoxy sections for electron microscopy was first introduced in the literature by Stirling and Graff (1995). The test tissue was glutaraldehyde and osmium-fixed cornea with crystalloid inclusions of IgG. Firstly, the sections were etched in saturated sodium metaperiodate for 1 h at room temperature in order to partially remove the osmium tetroxide from the sections. Then the same ultrathin sections of this tissue were exposed to antigen retrieval in 0.01M citrate buffer, pH 6.0 at 95 °C. The sections which were treated in this way obtained a significantly higher intensity of immunogold labeling with anti IgG than non-treated sections and sections which were treated in other conventional manners (see section IB). Crystalloid inclusions of IgG give very high concentrations of IgG in the tissue, and it is easier to obtain intense immunolabeling than if IgG occurs in less concentrated forms. This explains why these sections give intense immunolabeling even if the sections originally were fixed with osmium tetroxide [this fixative is very antigen-hostile (Baschong et al., 1984)]. When IgG occurs as immune complex deposits in osmicated glomerular tissue, the immunogold labeling is labeled in a weakly positive way (Brorson et al., 1999). Other early studies in the literature which used similar antigen retrieval techniques of epoxy sections were Wilson et al. (1996) and Brorson (1998b). Wilson et al. (1996) observed an increase following antigen heating from + to +++ with anti-Collagen V to basal lamina antigens in osmicated tissue. Brorson (1998b) obtained an immense increase in the immunogold labeling with anti-fibrinogen and anti-IgG to antigens in non-osmicated glomerular tissue as a consequence of heat-induced antigen retrieval. The increase (in per cent) from non-heated to heated epoxy sections is much larger than for acrylic sections (especially for non-osmicated tissue) and for paraffin sections for light microscopy (Brorson, 1998b, 1999a).

If nothing else is stated, the heating procedures in the following chapters are performed according to the method of Stirling and Graff (1995) at 95 °C.

II. Mechanisms for the increased intensity of immunolabeling following antigen retrieval of epoxy section by heating in aqueous solution

As mentioned in section IA, the anticipated mechanism for antigen retrieval on formaldehyde-fixed paraffin sections for light microscopy is breakage of formaldehyde-antigen bounds. This mechanism also has to be present when performing antigen retrieval on epoxy sections, since the same chemical bounds are present there (a combination of formaldehyde and glutaraldehyde is often used as fixative for immunoelectron microscopy - sometimes only formaldehyde). But since the increase (per cent) in the intensity of immunolabeling from non-heated to heated sections is much larger for epoxy sections than for acrylic sections and paraffin sections, other mechanisms have to be present. This has been studied by Brorson (1998b).

IIA. Breakage of epoxy protein bonds with formaldehyde as a link

When embedding tissues in epoxy resin copolymerization occurs between amino acid side groups of proteins and the resin polymer chains (Causton, 1984; Kellenberger et al., 1987), and this will result in fewer epitopes exposed at the surface of the epoxy sections than on the acrylic sections, especially for large proteins (Brorson and Skjørten, 1996a). It is thought that the reactions between amino acid side groups and the epoxy polymer may occur with the fixative as a link, while the epoxy resin may react directly with other protein side groups. When the tissue is fixed by aldehydes, side groups of proteins (especially $-NH_2$ groups) are occupied by the aldehyde fixative. Formaldehyde ($H_2C=O$) reacts with amino groups on the protein side chains (Griffiths, 1992) (Fig. 1a). This often occurs in a non-cross-linking way so the formaldehyde

molecules have one free reactive group (Fig. 1a). When the fixed tissue is embedded in epoxy resin, the epoxy chains may react with the new hydroxyl group introduced on the protein (Fig. 1b), and thereby the protein and the epoxy resin are copolymerized. Formaldehyde fixation is a reversible process (Hayat, 1989). When the epoxy sections are exposed to antigen retrieval by heating in citrate buffer, some of the bonds between the fixative and proteins will be hydrolysed (Hayat, 1989) (Fig. 1c). Oligomers of formaldehyde will also bind to the protein side groups, and these fixing molecules can be linked between the epoxy chains and the proteins and released in a similar way as proposed for monomeric formaldehyde (Hayat, 1989). We may speculate that glutaraldehyde acts in a similar way although glutaraldehyde fixation is less reversible. As a result of this breakage, the proteins are at least partly released from the epoxy network, and more surface epitopes are exposed to the immunoreagents because of superficial deplasticizing (Brorson, 1998b).

Fig. 1a

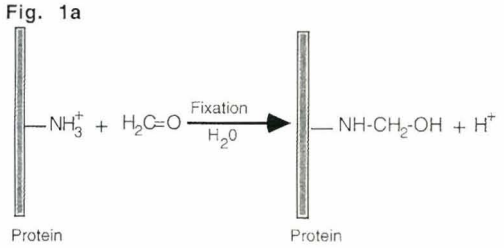

Fig. 1b

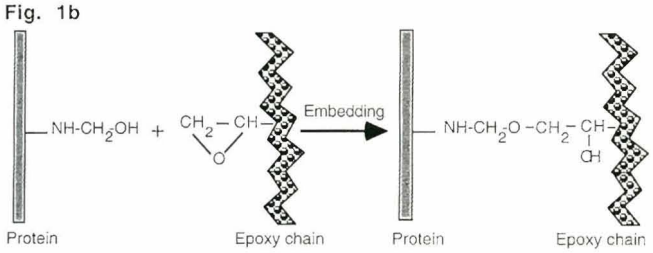

Fig. 1c

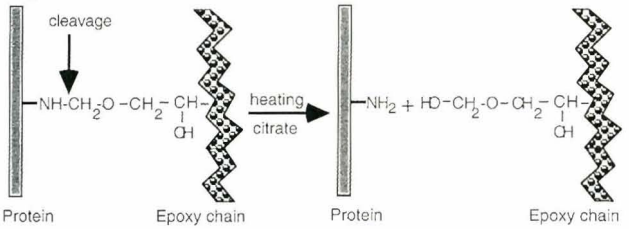

Fig. 1. a. The interaction between formaldehyde ($H_2C=O$) and the amino acid side groups (the basic amino acid lysine is especially exposed for binding to formaldehyde) of proteins in the fixation process. **b.** The epoxy resin binds to the protein side groups with the fixative as a link molecule. **c.** Formaldehyde fixation is a reversible process, and antigen retrieval by heating the epoxy sections in citrate solution may break the bonds between the fixative and the protein (Hayat, 1989), and since the fixative is a link between the protein and the epoxy resin, the protein will be partly released from the epoxy network. (Brorson, 1998b).

IIB. Breakage of direct epoxy-protein bonds

Even if the theory stated in section IIA is plausible, this is not the only mechanism by which increased immunolabeling following heating of epoxy sections occurs. It has been illustrated that disruption of direct epoxy-protein bounds (without formaldehyde as a link) occurs as a consequence of heating the epoxy sections in aqueous buffer solution (Brorson, 1999a).

When labeling epoxy-embedded immune complex deposits in ethanol-fixed renal tissue with anti-IgG and ethanol-fixed fibrin clots with anti-fibrinogen there were significant increases in immunogold labeling due to heating in citrate solution (up to 6-fold increase). This means that even when a non-aldehyde, ethanol, is used as the only fixative, there is an immense increase in the immunogold labeling after heating in citrate buffer.

Another observation by Brorson (1999a) which points in the same direction is that the relative increase in immunolabeling following heating in citrate buffer for epoxy sections of glutaraldehyde-fixed and formaldehyde-fixed tissue is approximately the same. If breakage of bonds following heating only occurred with the fixative as a link, the relative increase following heating should be significantly larger for formaldehyde-fixed tissue than glutaraldehyde-fixed tissue since formaldehyde-bonds are more reversible (Griffiths, 1992). Brorson (1999a) therefore hypothesized that heating of epoxy sections also may induce breakage of chemical bonds between the epoxy resin and the protein side groups in other ways than through the formaldehyde link.

IIC. Antigen retrieval of high and low accelerator epoxy sections

The observation that the increase of labeling in relative terms from non-retrieved epoxy sections to retrieved ones is larger the less accelerator used in the tissue processing was made by Brorson (1998b). This is thought to be caused by the presence of more chemical bonds between the epoxy resin and the tissue when low concentrations of accelerator are used in the tissue processing (Brorson and Skjørten, 1996c); when there are many bonds to break, there is a larger potential for increase in the immunolabeling. The same report shows more intense immunolabeling of retrieved high-accelerator epoxy sections than retrieved low-accelerator epoxy sections. This illustrates that breakage of epoxy-protein bonds in the low accelerator epoxy sections is not enough to surpass the combined effect of reduced copolymerization in high-accelerator epoxy sections and the breakage of some of its relatively few epoxy-protein bonds. This is explained in the following way: from theoretical considerations large proteins have large tendency to be cleaved during sectioning, and are more likely to be destroyed for immunodetection when embedded in low-accelerator epoxy resin than in high-accelerator epoxy resin (Brorson and Skjørten, 1996a,c).

This effect can never be compensated for by retrieval-breakage of epoxy-protein bonds (Brorson, 1998b).

IID. Antigen retrieval of acrylic sections and epoxy sections with differently fixed tissue

As mentioned in section I the relative increase of immunolabeling following antigen retrieval by heating in aqueous solution is significantly higher for epoxy sections than for acrylic sections. When tested on renal tissue with anti-IgG on immune complex deposits and fibrin clots with anti-fibrinogen, these relative increases for epoxy sections were in the range of 4-10 fold (or even higher), while for acrylic sections (LR-White) it was 1.25-2.2 fold. Similar results were observed for epoxy sections of ethanol-fixed tissue, but for acrylic sections no significant increase was observed on acrylic sections (Brorson, 1999a). These observations are caused by the fact that acrylic resins do not bind covalently to protein-side groups, and therefore acrylic sections have less to gain from the retrieval process - except for release of pure fixation bonds.

For epoxy sections the relative increase in immunolabeling following antigen retrieval by heating in aqueous solution is almost independent of the type of aldehyde used as fixative (formaldehyde or glutaraldehyde). For LR-white sections the relative increase in immunogold labeling (same antibodies and tissue as in the former paragraph) was significantly higher when the tissue was fixed with formaldehyde than when fixed with glutaraldehyde because formaldehyde-protein links are more reversible than the corresponding glutaraldehyde links (Brorson, 1999a). This also emphasizes that for epoxy sections, the effect of larger release of pure fixation bonds in the formaldehyde-fixed tissue than in the glutaraldehyde-fixed tissue is overflowed by the release of an immense number of fixative-independent protein-epoxy bonds.

III. Comparison of heat-induced antigen retrieval with other methods for immunoenhancing epoxy sections

As mentioned in the introduction Stirling and Graff (1995) were the first ones to introduce heat-induced antigen retrieval on epoxy sections in aqueous buffer (in combination with metaperiodate to remove osmium from the osmicated sections). This method was significantly better than the other methods that they performed simultaneously (different combinations of treatment with sodium ethoxide, metaperiodate). In this chapter we will consider comparison of heat-induced antigen retrieval with other and analogous techniques.

IIIA. Comparison of heat-induced antigen retrieval and etching with H_2O_2

Several explanations for H_2O_2-induced immunolabeling are proposed in the literature. Earlier, the most common one was that the epoxy sections become more hydrophilic after treatment with H_2O_2, and that this facilitates immunolabeling. But Dürrenberger et al. (1991) showed that the yield of immunolabeling of resin sections is independent of their hydrophilic or hydrophobic character. A more reliable theory proposes that the oxidation by H_2O_2 partly breaks epoxy resin bonds (Pfeiffer, 1982). Therefore, the accessibility of the epitopes can be increased on the surface of the section (Brorson, 1998a). Heating of epoxy sections increases the yield of immunolabeling by breakage of bonds between the epoxy resin and the antigens - where the fixative is sometimes a link as explained above (Brorson, 1998, 1999a). In this way it is a similar mechanism for the increased immunolabeling of H_2O_2-treated and heated epoxy sections.

When performing immunogold labeling with anti-IgG on glomerular immune complex deposits, Brorson et al. (2001) observed that the heated epoxy sections gave more than twice the yield of immunolabeling compared to the H_2O_2-treated sections (osmium-fixation was omitted). This shows that heating in citrate buffer is a more potent 'bond-breaker' than treatment with H_2O_2. When the epoxy sections are treated with H2O2, some bonds between epoxy resin and the antigens are broken. When H_2O_2-treated sections are heated in citrate buffer, more bonds are broken and the immunolabeling becomes more intense. But the bonds which were initially disrupted by the H_2O_2, would also have been broken by heating, and therefore the combination of heating and H_2O_2 does not give more intense immunolabeling than heating alone (Brorson et al., 2001).

Another study on glutaraldehyde and osmium-fixed tissue including amyloid AA shows significantly better immunolabeling with anti-AA for heated epoxy sections than for sections which were treated with H_2O_2 or sodium metaperiodate (Röcken and Roessner, 1999). We also have to take into consideration that H_2O_2 and sodium metaperiodate contribute to remove osmium from the sections which also facilitates immunolabeling. Röcken and Roessner (1999) claimed somewhat higher intensity of labeling when EDTA buffer was used as retrieval medium than for citrate buffer.

IIIB. Comparison of heat-induced antigen retrieval and deplasticizing with sodium ethoxide

More intense immunolabeling with anti-IgG on glomerular immune complex deposits for completely deplasticized epoxy sections (deplasticized by sodium ethoxide) than for heated epoxy sections (both for epoxy sections based on embedding with low and high concentration of accelerator) was observed by Brorson (1999b). This result is reasonable since all resin-protein bonds are broken during the complete deplasticizing, while only some of the epoxy-protein bonds are broken during heat-induced antigen retrieval. The release of fixation bonds following heating in citrate buffer does not give so much increase in immunolabeling that it

compensates for the lack of complete absence of epoxy-protein bonds on the heated sections. But on the other hand, the ultrastructural preservation of the tissue is far better on the heated sections. Besides, it is possible to perform immunogold labeling of heated epoxy sections on both sides of the sections if we omit the formwar coat on the grids, and this gives a potential 2-fold increase in the immunolabeling compared to the earlier statement in this paragraph. Such two-sided immunolabeling is not possible on completely deplasticized epoxy sections, since the sections then will be entirely dissolved (Brorson, 1999, 2001). If we perfom non-complete deplasticizing (only 'etching' with ethoxide) of the epoxy sections, we do not necessarily obtain more intense immunolabeling of ethoxide-treated sections than heated sections (Stirling and Graff, 1995; Brorson, 2001).

IIIC. Comparison of heat-induced antigen retrieval, high-accelerator epoxy sections and acrylic sections

As mentioned in the introduction, we can obtain a higher level of immunogold labeling on epoxy sections if we use higher concentrations of accelerator when infiltrating and embedding the tissue, and this enhanced immunolabeling occurs without the heating procedure (Brorson and Skjørten, 1996a,c; Brorson et al., 1997). But even if we heat low-accelerator epoxy sections, reports show that it will obtain approximately the same immunolabeling as for non-heated high-accelerator sections (Brorson, 1998b). Difficulties with infiltration of some tissues, low contrast of the sections and problems with trimming the blocks are obstacles for the high-accelerator method, but these problems are not present when heating low/moderate-accelerator epoxy sections (Brorson and Strøm, 1998; Brorson et al., 1999).

When we combine high-accelerator epoxy sections with heating (se section IIC), we may obtain a degree of immunolabeling which is higher than for non-heated LR-white sections (Brorson, 1998b), and the problem with low stainability of the high-accelerator epoxy sections almost vanishes when they are heated beforehand. But if we compare heated high-accelerator epoxy sections with heated LR-white sections, the last ones are significantly more intensely labeled (Brorson, 1998b; 1999b). Heated high-accelerator epoxy sections also tend to have somewhat higher background staining on the resin.

IV. How does the effect of heat-induced antigen retrieval depend on the temperature in the retrieval medium?

All that has been written until now in this review about heat-induced antigen retrieval, is based on heating the sections to temperatures just below the normal boiling point of water (≈ 95 °C). What happens if we increase the temperature during the retrieval far beyond this temperature? To obtain higher temperature in an aqueous solution, we have to use an autoclave or pressure cooker where the pressure can be increased beyond 1 atm. Autoclave-heating of formaldehyde-fixed paraffin sections is sometimes used (Bankfalvi et al., 1994; Bier et al., 1995; Taylor et al., 1996; Mote et al., 1998), but not as frequently as microwave heating. For paraffin sections, some researchers report approximately the same intensity of immunolabeling with autoclave heating as for microwave heating just below 100 °C (Taylor et al., 1996), while other reports show that autoclave treatment gives a significantly higher level of immunolabeling (Bankfalvi et al., 1994; Bier et al., 1995; Mote et al., 1998). Autoclave pretreatment at 121 °C of acrylic sections has been reported to give approximately the same intensity of immunogold labeling as when heating the sections just below the normal boiling temperature (Xiao et al., 1996). Brorson and Nguyen (2001) have recently performed immunogold labeling of non-osmicated epoxy sections which have been exposed to antigen retrieval in the range of 25 °C to 135 °C with steps of approximately 10 °C. The interesting results and conclusions from this work are presented in the rest of this section.

The immunogold labeling was performed with anti-

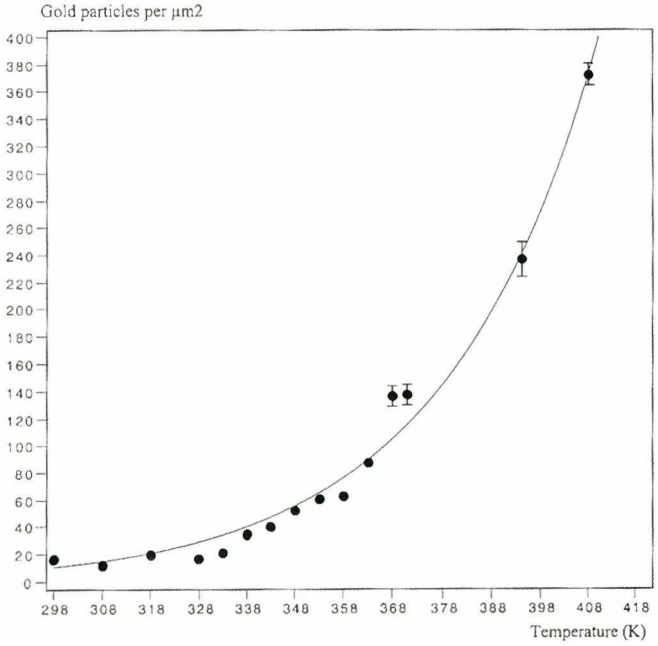

Fig. 2. The intensity of immunolabeling with anti-IgG (as gold particles per μm2) on immune complex deposits in glomerular tissue on epoxy sections autoclave heated in citrate buffer at temperatures ranging from 25 °C to 135 °C (that is 298-408 Kelvin (K)). This figure shows exponential growth in the intensity of the immunogold labeling as a function of the temperature by the "best fit"-formula:

$$y_{IgG} = 9.38 \cdot 10^{-4} e^{0.0316 \cdot x}$$

Similar exponential growth is present for labeling with anti-C3. (Brorson and Giang, 2001).

IgG and anti-C3 on immune complex deposits in glomerular tissue on moderate-accelerator epoxy sections. Increasing the temperature from 95 °C to 135 °C in the retrieval medium (citrate buffer) yieded a significant increase in the intensity of immunogold labeling; 2.8-fold increase for anti-IgG and 11-fold increase for anti-C3. There were significant differences in the effect of the antigen retrieval depending on the temperature in the autoclave (Fig. 2): heating the sections at 135 °C gave significantly higher level of immunogold labeling compared with heating at 121 °C. With antigen retrieval at 135 °C the level of IgG-labeling of epoxy sections was estimated to be only 15% lower than the corresponding labeling of LR-White sections heated at 95 °C. The ultrastructural preservation of the tissue was not significantly disturbed as a result of the extreme temperature in the autoclave.

When the intensity of the immunolabeling is considered as a function of the temperature in the range of 25-135 °C, there was an exponential growth in this area for immunolabeling both with anti-IgG and anti-C3 (Fig. 2).

The observation that the level of immunogold labeling increased when the temperature of the citrate buffer increased, can be explained in the following way: retrieval media with higher temperatures have larger ability to disrupt the bonds between the epoxy resin and the antigens. It is believed that antigens located at a level below those at the surface of the section may then protrude as a result of this bond-breaking and epoxy resin etching created by the heat. Therefore, these new antigens will then be available for interaction with the immunoreagents after the antigen retrieval process. None of these newly protruding antigens may have been damaged in the cutting process, while some of the antigens originally located at the surface have been cleaved by the knife during the cutting process. (Brorson and Nguyen, 2001).

V. Speculation: are heat-induced antigen retrieval of epoxy sections and deplasticizing strictly speaking the same techniques with a similar mechanism? how does this influence further improvement?

Deplasticizing

Complete removal of epoxy resin from the sections by strong sodium ethoxide is caused by the ability of the strong alkaline solution to disrupt the ester bonds of the epoxy resin network (Mar and Wight, 1988; Baigent and Müller, 1990; Brorson and Skjørten, 1995). The strong alkali solution works as a catalyst in this process. The immunolabeling is strongly enhanced by this process (formwar-coated grids have to be used to avoid complete loss of sections). If we use a diluted solution of sodium ethoxide (1%) for 10 min at 22 °C, the sections will not be dissolved from a non-coated grid, and the immunolabeling is still significantly enhanced, but not so much as for the completely deplasticized sections (Brorson, 2001).

Heat-induced antigen retrieval

When we use citrate-solution with pH 6 as the antigen retrieval medium at 135 °C, there is no problem with dissolving of the epoxy sections from the non-coated grid. If we increase the pH in the citrate solution to 10-12 at the same temperature, the epoxy sections are completely dissolved in the retrieval process. At pH 9 we observe more extracted and thinner sections (personal experience).

Speculation on the mechanism

On the basis of the last paragraphs we may speculate that the mechanism for both heat-induced antigen retrieval and deplasticizing of epoxy sections is the epoxy-ester bond's tendency to dissolve in aqueous solution. When performing deplasticizing, strong alkali is the catalyst. When performing heat-induced antigen retrieval, heat is the catalyst. We also may have a combination of alkaline and heat catalysing for both techniques. When performing heat-induced antigen retrieval at pH 6 at 135 °C or deplasticizing with 1% of saturated sodium ethoxide solution (Brorson, 2001) we obtain a partial and superficial deplasticizing which increases the exposure of the antigens to the immunoreagents. But the ethoxide technique performs more deplasticizing throughout the sections which destroys the sections if the same degree of two-sided immunolabeling is to be achieved. The ability of hot citrate solution to break formaldehyde bonds between protein, gives an extra tiny contribution to the increase in immunolabeling with the heat-retrieval method.

If these speculations are correct, we can expect at best a tiny gain in the intensity of the immunolabeling by combining heat-induced antigen retrieval in the autoclave and deplasticizing, since the superficial deplasticizing is already performed by the heat treatment (in general, antibodies do not penetrate deplasticized epoxy sections [Brorson and Skjørten, 1995]).

VI. Concluding remarks

Traditionally, epoxy sections have not been regarded as well-suited for immunoelectron microscopy. This view ought to be reconsidered in the light of the progresses of heat-induced antigen retrieval, and it has to be emphasised that it is the non-treated epoxy sections which is often incompatible with immunoelectron microscopy. Autoclave-heating of epoxy sections is easy to perform, especially if a small, low cost laboratory autoclave (≈ 1000$) is used. Therefore, heat-induced antigen retrieval at high autoclave temperature, which may give almost the same intensity of immunolabeling as acrylic sections heated at normal temperatures, is expected to give a boost to the use of epoxy sections for immunoelectron microscopy.

References

Baigent C.L. and Müller G. (1990). A new approach to the immunostaining of epoxy-resin-embedded material. J. Microscopy 158, 73-80.

Bankfalvi A., Navabi H., Bier B., Böcker W., Jasani B. and Schmid K.W. (1994). Wet autoclave pretreatment for antigen retrieval in diagnostic immunohistochemistry. J. Pathol. 174, 223-228.

Baschong W., Baschong-Prescianotto C., Wurtz M., Carlemalm E., Kellenberger C. and Kellenberger E. (1984). Preservation of protein structures for electron microscopy by fixation with aldehydes and/or OsO_4 (Osmium). Eur. J. Cell Biol. 35, 21-26.

Bier B., Bankfalvi A., Grote L., Blasius S., Öfner D., Böcker W., Jasani B. and Schmid K.W. (1995). Wet autoclave pretreatment for immunohistochemical demonstration of oestrogen receptors in routinely processed breast carcinoma tissue. Histochem. J. 27, 148-154.

Boon M.E. and Kok L.P. (1994). Microwaves for immuno-histochemistry. Micron 25, 151-170.

Brorson S.H. (1997). How to examine the antigen-damaging effect of sodium ethoxide on deplasticized epoxy sections. J. Histochem. Cytochem. 45, 143-146.

Brorson S.H. (1998a). Antigen detection on resin sections and methods for improving the immunogold labeling by manipulating the resin. Histol. Histopathol. 13, 275-281.

Brorson S.H. (1998b). The combination of high-accelerator epoxy resin and antigen retrieval to obtain more intense immunolabeling on epoxy sections than on LR-White sections for large proteins. Micron 29, 89-95.

Brorson S.H. (1999a). The fixative-dependent increase in immunogold labeling following antigen retrieval on acrylic and epoxy sections. Biotech. Histochem. 74, 248-260.

Brorson S.H. (1999b). Comparison of the immunogold labeling of heated and deplasticized epoxy sections on the electron microscopical level. Micron 30, 319-324

Brorson S.H. (2001). Deplasticizing or etching of epoxy sections with different concentrations of sodium ethoxide to enhance the immunogold labeling. Micron 32, 101-105.

Brorson S.H. and Nguyen G.H. (2001). Increased level of immunogold labeling of epoxy sections by rising the temperature significantly beyond 100 °C in the antigen retrieval medium. Micron 32, 591-597.

Brorson S.H. and Skjørten F. (1995). Mechanism for antigen detection on deplasticized epoxy sections. Micron 26, 301-310.

Brorson S.H. and Skjørten F. (1996a). The theoretical relationship of immunogold labeling on acrylic sections and epoxy sections. Micron 27, 193-201.

Brorson S.H and Skjørten F. (1996b). The theoretical ratio of immunogold labeling of deplasticized epoxy sections and acrylic sections. Micron 27, 203-209.

Brorson S.H. and Skjørten F. (1996c). Improved technique for immunoelectron microscopy. How to prepare epoxy resin to obtain approximate the same immunogold labeling for epoxy sections as for acrylic sections without any etching. Micron 27, 211-217.

Brorson S.H. and Strøm E.H. (1998). A new immunoelectron microscopy approach for the detection of immunoglobulin and complement deposits in epoxy-embedded renal biopsies. Ultrastruct. Pathol. 22, 449-457.

Brorson S.H., Strøm E.H. and Skjørten F. (1997). Immunoelectron microscopy on epoxy sections without deplasticizing to detect glomerular immunoglobulin and complement deposits in renal diseases. APMIS 105, 139-149.

Brorson S.H., Andersen T., Haug S., Kristiansen I., Risstubben A., Tchou H. and Ulstein J. (1999). Antigen retrieval on epoxy sections based on tissue infiltration with moderately increased amount of accelerator to detect immune complex deposits in glomerular tissue. Histol. Histopathol. 14, 151-155.

Brorson S.H., Hansen A.R., Nielsen H.Z. and Woxen I.K. (2001) A comparative study of the immunogold labeling on H_2O_2-treated and heated epoxy sections. Micron 32, 147-151.

Causton B.E. (1984). The choice of resins for electron immunocytochemistry. In: Immunolabeling for electron microscopy. Polak J.M. and Varndell I.M. (eds). Elsevier Science Publishers. Amsterdam, New York. pp 29-36.

Dürrenberger M., Villiger W., Arnold B., Humbel B.M. and Schwarz H. (1991). Polar or apolar lowicryl resin for immunolabeling. In: Colloidal gold: Principles, methods and applications. Vol. 3. Hayat M.A. (ed). Academic Press. pp 73-85.

Griffith G. (1992). Fine structure preservation. In Fine Structure Immunocytochemistry. Springer Verlag. Berlin. pp 36-51.

Hayat M.A. (1989). Chemical fixation. In: principles and techniques of electron microscopy. The Macmillan Press. London. pp 37-40

Holm R., Farrants G.W., Nesland J.M., Sobrinho-Simoes M., Jørgensen O.G. and Johannessen J.V. (1989). Ultrastructural and electron immunohistochemical features of medullary thyroid carcinoma. Virchows Arch. (A) 414, 375-384.

Kellenberger E., Dürrenberger M., Villiger W., Carlemalm E. and Wurtz M. (1987). The efficiency of immunolabel on Lowicryl sections compared to theoretical predictions. J. Histochem. Cytochem. 35, 959-969.

Mar H. and Wight T.N. (1988). Colloidal gold immunostaining on deplasticized ultra-thin sections. J. Histochem. Cytochem. 36, 1387-1395.

Morgan J.M., Navabi H., Schmid K.W. and Jasani B. (1994). Possible role of tissue-bound calcium ions in citrate-mediate high-temperature antigen retrieval. J. Pathol. 174, 301-307.

Mote P.A., Leary J.A. and Clarke C.L. (1998). Immunohistochemical detection of progesterone receptors in archival breast cancer. Biotech. Histochem. 73, 117-127.

Newman G.R. (1989). LR-White embedding medium for colloidal gold methods. In: Colloidal Gold: principles, methods and applications. Vol. 2. Hayat M.A. (ed). Academic Press. pp 47-73.

Pfeiffer S.W. (1982). Use of hydrogen peroxide to accelerate staining of ultrathin spurr sections. Stain Technol. 57, 137-142.

Röcken C. and Roessner. (1999). An evaluation of antigen retrieval procedures for immunoelectron microscopic classification of amyloid deposits. J. Histochem. Cytochem. 47, 1385-1394.

Shi S.R., Key M.E. and Kalra K.L. (1991). Antigen retrieval in formalin-fixed paraffin-embedded tissue: An enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. J. Histochem. Cytochem. 39, 741-748.

Stirling J.W. and Graff P.S. (1995). Antigen unmasking for immunoelectron microscopy: labeling is improved by treating with sodium ethoxide or sodium metaperiodate, then heating on retrieval medium. J. Histochem. Cytochem. 43, 115-123.

Taylor C.R., Shi S.R., Chen C., Young L., Yang C. and Cote R.J. (1996). Comparative study of antigen retrieval heating methods: microwave, microwave and pressure cooker, autoclave, and

steamer. Biotech. Histochem. 71, 263-270.

Wilson D.F., Jiang D.J., Pierce A.M., Wiebkin O.W. (1996). Antigen retrieval for electron microscopy using a microwave. Appl Immunohistochem. 4, 66-71.

Xiao J.C., Adam A., Ruck P. and Kaiserling E. (1996). A comparison of methods for heat-mediated antigen retrieval for immunoelectron microscopy: demonstration of cytokeratin No. 18 in normal and neoplastic hepatocytes. Biotech. Histochem. 71, 278-285.

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