Effect of EDTA on Cytokeratin Detection in the Inner Ear

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Immunohistochemical studies on the epithelium of the adult inner ear are difficult to perform without decalcification of the bony capsule. In this study, we examined the effect of decalcifying agents on the immunoreactivity of various cytokeratin antigens in the cochlear duct epithelium of 2-day-old rats, allowing the comparison of fresh and decalcified specimens. Decalcification of unfixed tissue in a solution containing EDTA or EGTA and polyvinylpyrrolidone, at pH 7.4 and 4°C for a maximum period of 2 days, not only preserved the antigen epitopes but even enhanced the staining intensities in comparison with fresh specimens. This enhancement effect, caused by chelating agents and found to be blocked by prior fixation with acetone, is suggested to be caused by unmasking of the antigenic epitopes. (J Histochem Cytochem 38:1223–1227)

RE: Words: Inner ear epithelium; Cytokeratin; EDTA; EGTA; Unmasking; Fixation; Immunohistochemistry.

Introduction
During recent years, immunohistochemical staining procedures have become important tools for the identification of cellular antigens. Because of the widely differing natures and structures of the antigens to be localized, this method can present problems for the investigator, and because of the nature of fixation, alterations in protein structure and antigenicity may produce false-positive or false-negative results. For example, some antigens can be localized only in frozen sections of fresh tissue, whereas others are still reactive after gentle fixation and processing.

The results of several studies have shown that uniform fixation and processing protocols can be followed when cells and tissues are to be examined by means of immunohistochemical procedures (1,4,6). Depending on the nature and structure of the antigens, appropriate protocols are required to prevent destruction or masking of the antigenic epitopes.

Additional problems must be overcome if antigens are to be localized in hard tissues containing bone. No systematic studies are available that describe the effects of decalcification on specific antigens. We were faced with this problem when we attempted to document the expression of cytokeratin polypeptides in the epithelia of the inner ear with the use of monoclonal antibodies. In the adult, the organ is completely surrounded by bone, and dissection of these delicate structures often causes loss of or damage to the epithelia. Therefore, decalcification of the bony capsule is desirable.

From the sparse data reported on the effect of decalcifying agents on tissue antigens (13,14,17), it can be concluded that decalcification in EDTA (ethylene diamino tetraacetic acid) appears to be quite suitable for the demonstration of some surface antigens and antigens in the extracellular matrix of bone. However, no data are available concerning the effect of EDTA on the detection of cytokeratin polypeptides in general, and in the developing cochlear duct of the inner ear of young rats in particular.

To evaluate the effect of chelating agents, we chose to use 2-day-old rats, because at that age ossification of the skull is limited, which allows cryosectioning of non-decalcified specimens. This enabled us to compare fresh and decalcified specimens.

Materials and Methods
This study was performed on 2-day-old Wistar rats. After decapitation, the inner ear was quickly dissected and either immediately frozen in liquid nitrogen or transferred to one of the following media.

Medium 1: 0.1 M Tris-HCl buffer (pH 7.4; Merck, Darmstadt, FRG) containing 10% EDTA (disodium salt; Merck) and 7.5% polyvinylpyrrolidone (PVP, cryoprotectant; Serva, Heidelberg, FRG) (13).

Medium 2: as 1, but containing EGTA (ethylene glycol tetraacetic acid, Sigma, St. Louis, MO) instead of EDTA.

Medium 3: as 1, but without EDTA.

Medium 4: 0.1 M phosphate buffer (pH 7.4; BDH, Montreal, Canada) containing 1% paraformaldehyde (Merck) and 0.1% glutaraldehyde (Merck) (10) for 2 hr, followed by storage in either Medium 1 or 3.

Medium 5: 0.04 M phosphate buffer (pH 6.2) containing 0.1 M lysine-HCl (Sigma), 0.01 M Ns-metamidodote (Sigma), and 2% paraformaldehyde (modified after McLean and Nakane (15)) for 2 hr, followed by storage in Medium 1.

Medium 6: Medium 1 or 3 containing the following protease inhibitors: trypsin (0.5 mg/100 ml; Sigma), leupeptin (0.5 mg/100 ml;
Sigma), and diisopropylfluorophosphate (1.74 mg/100 ml; Sigma), according to Aronson and Greenbaum (2).

After a storage period ranging from 1 hr to 7 days (at 4°C), all the specimens were rinsed in 0.1 M Tris-HCl buffer (pH 7.4) containing 7.5% FVP at 4°C for 4 hr and subsequently frozen in liquid nitrogen.

Frozen sections (7 μm) were placed on poly-L-lysine (Sigma)-coated microscope slides. They were allowed to dry at room temperature and were stored at −70°C, ready for staining with the indirect immunoperoxidase technique. For this purpose, the sections were fixed in cold acetone (Merck) at 4°C for 5 min, rinsed in PBS, and incubated at room temperature for 45 min with primary monoclonal antibodies directed against cytokeratins. After washing in PBS (three times for 10 min each), the sections were incubated for 30 min with peroxidase-conjugated rabbit anti-mouse immunoglobulin (diluted 1:40 in PBS; Dakopatts, Glostrup, Denmark), containing 5% (v/v) normal rat serum (Dakopatts). After washing in PBS (three times for 10 min each), followed by Na-acetate buffer (0.05 M, pH 4.9; Merck) (two times for 3 min each), the peroxidase activity was detected by a 10-min treatment with AEC (20 mg of 3-amin-9-ethylcarbazole (Sigma) in 100 ml of 0.05 M Na-acetate buffer, pH 4.9 (Merck)) containing 5 ml dimethylformamide (DBH) and 0.01% hydrogen peroxide (Merck).

After rinsing in distilled water, the sections were counterstained with Mayer's hemalum and mounted in gelatin jelly. As a control, the first antibody was omitted.

From the group of specimens that were freshly frozen before any treatment, sections were exposed to one of the media quoted under 1, 2 or 3, for 5–30 min and subsequently washed in PBS (three times for 10 min each) before incubation with the primary antibodies. To evaluate the influence of acetone on the effect of chelating agents, acetone fixation (5 min at 4°C) of these sections was performed either before or after exposure to the media.

Sections that had been taken from fixed tissues (Media 4 and 5) were treated with trypsin (0.1%; Sigma) in 0.05 M Tris-HCl buffer (pH 7.8) or neuraminase (0.1%; Sigma) in PBS (pH 7.3) for 10 min at 37°C, to recover the antigenic epitopes (3), and subsequently washed in PBS (three times for 0.3 min each) before incubation with the primary antibodies.

The following monoclonal antisera, mainly recognizing individual cytokeratins, were used in this study, either undiluted or diluted with PBS:

(a) RCK 102 (undiluted) directed against cytokeratins 3 and 8. This antibody stains virtually all epithelial cells; (b) RCK 103 (diluted 1:5) directed against cytokeratin 7. This antibody stains most transitional epithelia and a subpopulation of glandular epithelium; (c) RGE 53 (diluted 1:6) directed against cytokeratin 18; (d) CK 18-2 (undiluted) directed against cytokeratin 18; and (e) LP2K (undiluted) directed against cytokeratin 19. These antibodies specifically stain most of the columnar epithelia.

All of these antibodies, raised against human proteins, have been described in previous studies (5,15,18). Although they have been tested mainly on human tissues and the nomenclature is that of human cytokeratins, a similar specificity has been demonstrated in various rat tissues (11,19,23).

For all the different treatments, four to six ears were tested. Each antibody was tested on an average number of five sections, mainly midmodiolar, containing all cochlear turns. Micrographs were taken from the second turn.

Results
The cochlea consists of a fluid-filled triangular coiled duct which is lined with different types of epithelia. A micrograph of a routinely prepared glycol methacrylate section is included for general orientation (Figure 1), because the structural differences in the epithelial lining of the cochlea are not clearly visible on unfixed sections. Immunoperoxidase staining of cryostat sections taken from freshly frozen tissue, using monoclonal antibodies against various cytokeratins, revealed moderate staining of the epithelium of the stria vascularis, Reissner's membrane, interdental cells, and the basal part of the inner sulcus cells with the antibodies LP2K (Figure 2) and RCK 102. Weak staining with these antibodies was found in some cells of the organ of Corti. With CK 18-2, moderate staining was observed in the stria vascularis, whereas the organ of Corti, external sulcus, and Reissner's membrane stained very weakly. The remaining epithelia were negative (Table 1).

With RGE 53 all epithelia were negative, while with RCK 105

![Figure 1](image1.png)  
Figure 1. Micrograph of a routinely prepared glycol methacrylate section of the cochlear duct of a 2-day-old rat. This toluidine blue-stained section shows the different epithelia. ES, external sulcus; ID, interdental cells; IS, inner sulcus; O, organ of Corti; RM, Reissner's membrane; SV, stria vascularis. Bar = 50 μm.

![Figure 2](image2.png)  
Figure 2. Immunohistochemical staining of cryostat section taken from freshly frozen cochlear duct epithelium of a 2-day-old rat with the monoclonal antibody LP2K. Bar = 50 μm.
Table 1. Immunoreactivity of various monoclonal cytokeratin antibodies in fresh and EDTA-stored cochlear duct epithelium

<table>
<thead>
<tr>
<th>Antigen</th>
<th>RCK 102 (CK 5 + 8)</th>
<th>LP2K (CK 19)</th>
<th>CK 18-2 (CK 18)</th>
<th>RGE 53 (CK 18)</th>
<th>RCK 105 (CK 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fresh</td>
<td>EDTA</td>
<td>Fresh</td>
<td>EDTA</td>
<td>Fresh</td>
</tr>
<tr>
<td>Stria vascularis</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Reissner's membrane</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>External sulcus</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Organ of Corti</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Inner sulcus</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Lamina spiralis</td>
<td>+</td>
<td>+</td>
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<td>+</td>
</tr>
</tbody>
</table>

* Staining intensity: -, absent; +, weak; ++, moderate; ++++, strong.

only a very weak reaction was found in the external sulcus (Figures 3A and 3C).

Immunoperoxidase staining of sections obtained from tissues stored for 1 or 2 days in the medium containing EDTA revealed a strong positive reaction with RGE 53 in the stria vascularis and a weaker but distinct reaction in Reissner's membrane and the organ of Corti (Figure 3B). The stria vascularis now showed a distinct positive reaction with RCK 105 (Figure 3D). The staining with the antibodies RCK 102, CK 18-2, and LP2K was more intense than in the fresh sections (Table 1). Prolonged storage of the specimens in the media containing EDTA or EGTA for more than 2 days resulted in a gradual decrease in cytokeratin detectability. The struc-
The epithelial integrity of the specimens appeared to be well preserved at the light microscopic level for up to 5 days, except for a time-dependent shrinkage. Sections of specimens stored in a medium without chelating agents or with protease inhibitors failed to show any difference in expression from sections taken from untreated freshly frozen specimens. If the specimens were fixed in paraformaldehyde or glutaraldehyde before exposure to decalcifying agents, no staining reaction was found with the exception of RCK 102, which showed a weak reaction.

A further analysis of the effects of the various media on the expression of calcium staining was performed on mounted sections taken from freshly frozen tissues. Comparable results could be obtained if the sections were exposed to the media containing chelating agents. It appeared that there was an enhancement of detectability after 5-min incubation with the media containing EDTA or EGTA. However, these effects were completely absent when the sections were fixed for a few minutes in acetone before incubation with these media.

**Discussion**

This study demonstrates that fixation procedures which have been shown to leave different antigens intact (10,16,17,20) may have a destructive effect on the cytotkeratin antigens in the epithelial lining of the developing cochlear duct of the rat. Certain epitopes on these cytoskeletal components were found to resist only short fixation in acetone. After fixation in paraformaldehyde- or glutaraldehyde-containing media, only the RCK 102 epitopes were left partially intact.

Decalcification of unfixed tissues with EDTA (pH 7.4) at 4°C revealed fairly well-preserved structural integrity of the epithelial lining of the cochlear duct at this stage of development. A comparable observation on some other tissues has recently been reported by Stosiek et al. (21). In addition, it was found that the epitopes recognized by the antibodies tested were left intact if the exposure to the chelating agents did not exceed 2 days. Storage for longer periods resulted in a gradual loss of antigen detectability, presumably caused by extraction or proteolytic degradation of the antigens. Surprisingly, exposure of the inner ear epithelium to EDTA not only left the epitope sites intact but even had an enhancing effect on the detection/detectability of the cytokeratins tested. This resulted in the finding that EDTA and EGTA treatment revealed the presence of cytokeratins 7 and 18 at sites where they could not be detected in fresh sections, at least not by means of antibodies RCK 105 and RGE 53, respectively.

The lack of staining of distinct parts of the epithelial lining with some of the antibodies applied must be attributed to a different configuration of the cytokeratins in the various epithelia. In addition, the differing staining pattern obtained with the two CK 18 antibodies (CK 18-2 and RGE 53) is probably due to a different configuration of the epitopes recognized by these antibodies, as has been suggested by Franke et al. (9). EDTA chelates several different divalent cations, whereas EGTA preferentially binds calcium. The similarity between the effects induced by EDTA and EGTA enables us to conclude that removal of calcium from the tissue appears to be the main causative factor for the enhanced staining reactions.

Because calcium is an important co-factor in endopeptidase activity and has particularly been observed to activate a Ca²⁺-dependent protease specific to intermediate filament proteins (8,22), it might be suggested that the enhancement effect is due to inhibition of enzymatic destruction of the antigen receptor sites. Such an explanation seems unlikely, because incubation in media containing various endopeptidase inhibitors failed to produce any effect on the detection and staining of the two particular cytokeratins. Moreover, if endopeptidase activity is involved in antigen destruction, it should be especially pronounced in sections obtained from tissues stored in the media not containing chelating agents. However, in these sections enhanced cytokeratin staining could still be evoked through short exposure to EDTA or EGTA. Therefore, unmasking of the antigen epitopes by the chelating agents must be considered a more likely explanation, especially in view of the fact that the monoclonal antibody CK 18-2, which is also specific for cytokeratin 18, reacted with the epithelia irrespective of the pretreatment method. It has been demonstrated that aqueous solutions of EDTA at near-neutral pH not only remove calcium ions from bone matrix and cells but also remove a large proportion of the macromolecules consisting of non-collagenous proteins and carbohydrates (7,12). It seems logical that extraction of these and possibly other components also occurs in soft tissues and subsequently improves the access of antibodies to the antigens. Support for this assumption can be derived from the absence of enhancement when sections obtained from fresh tissue were fixed in acetone before incubation with chelating agents, whereas acetone fixation after treatment with these agents had no effect. Acetone exposure appears to leave the antigen epitopes intact but precipitates the macromolecular fraction, rendering it less susceptible to extraction. Furthermore, conformational changes in the heterotypic complexes of cytokeratin 8 and 18 and possibly also of cytokeratins 7 and 19, with or without the two former compounds, may be induced by EDTA and EGTA and thus unmask certain epitopes that are undetectable under normal physiological conditions (9).

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