Improved Staining of Negative Binding Sites with Ruthenium Red on Cryosections of Frozen Cells

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Abstract. Hexavalent cationic dye ruthenium red (RR) binds to anionic sites of cellular components, predominantly to the surface coat rich in glycoconjugates, and can be used as a marker of negative binding sites. Due to limited penetration of RR only superficial layers of cells are stained satisfactorily. To improve RR staining of L1210 leukemic cells isolated from culture and concentrated by centrifugation, cryosections of frozen cells were treated by RR to expose simultaneously all the cells and their components to the dye treatment. Cells were fixed with 2% glutaraldehyde in cacodylate buffer (CB), soaked in 2.2 mol/l sucrose and frozen by plunging into liquid nitrogen. Ultrathin cryosections were cut at a temperature of −90°C, transferred to Formvar coated copper grids, postfixed with 1% OsO_4 and stained with 0.05% RR in CB for 60–120 min. After removing RR solution with filter the grids were dried and examined electron microscopically. The resulting staining was a combination of a negative contrast (the plasma membrane and membranes of intracellular organelles) and of a positive contrast (cytoplasmic matrix and the extracellular coat). RR staining of negative binding sites on cryosections has proved useful for uniform exposure of all cells and cellular compartments to the dye and especially of external coat containing glycoconjugates.

Key words: Ruthenium red — Cryosections — Cell coat

Introduction

Polycationic dye ruthenium red (RR) stains negative binding sites in external coats of cells related to transport, barrier, or receptive functions, and has been used as a marker of glycoconjugates in cells of different origin (Luft 1964, 1971a,b; Vorbrodt and Koprowski 1969; Rosenfeld et al. 1973). The cell coat is rich in carbohydrates incorporated into glycoproteins, glycolipids, and proteoglycans carrying a negative charge at physiological pH.

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However, RR penetration into deeper parts of tissue (e.g. epithelial or endothelial layers) or cells (e.g. T-tubules of muscle fibres) is limited due to self-imposed restriction resulting from the repulsive forces of those RR^{6+} polycations already bound to cellular matrix containing anionic sites (Snowdowne and Howell 1984; Zacharová et al. 1990). This problem may severely limit the use of RR especially in studies of staining properties of cells isolated from tissues or cultures and concentrated by centrifugation.

The conventional double fixation and RR staining using glutaraldehyde with subsequent osmium tetroxide/RR mixture links the cells together and allows the RR binding only to the superficial layer of a sediment consisting frequently from debris of damaged cells. The fixation with osmium tetroxide/RR mixture alone, to avoid linking effect of glutaraldehyde, results in insufficient preservation of cellular components with the loss of material.

In the present study a new method of RR staining of negative binding sites on cryosections of glutaraldehyde-fixed frozen cells has been introduced with the aim to expose simultaneously all the cells and their components to the cationic dye treatment. The choice of the cells (L1210 mouse leukemic cell line) has been determined by our interest in the change of surface properties of these cells following the development of P-glycoprotein multidrug resistance (Kvačkajová-Kišucká et al. 2001; Kišucká et al. 2001; Drobná et al. 2002).

Materials and Methods

The mouse leukemic cell line L1210 was used. Cells were cultivated in RPMI medium containing 5 % fetal bovine serum. After cultivation cells were centrifuged at $1000 \times g$ for 3 min, washed in physiological saline and fixed with 2 % glutaraldehyde in 0.1 mol/l Na-cacodylate buffer (CB) at pH 7.2 for 60 min. During fixation the cells were centrifuged at $1000 \times g$ for 3 min to form a sediment. The specimen was then washed 3× for 7 min with CB and soaked in 2.2 mol/l sucrose in CB for 24 h at a room temperature. Small pieces of sediment were then transferred to copper pins and frozen by plunging into liquid nitrogen. Ultrathin (80 to 100 nm) cryosections were cut with glass knives at a temperature of −90 to −95 °C in a Reichert Ultracut E ultramicrotome equipped with FC 4D cryoattachment and transferred with a wire-loop containing a drop of 2.2 mol/l sucrose in CB to a Formvar coated copper grid. The grid was then washed on a drop of pure CB and then fixed on a drop of 1 % OsO_4 in CB for 30 min. After rinsing in the buffer the grids with sections were placed on a drop containing 0.05 % RR in CB and stained for 60–120 min. After removing RR solution with filter, the grids were dried and examined in a JEOL JEM 1200 EX electron microscope at 80 kV.

Results

Fig. 1A shows the result of conventional double fixation and RR staining using glutaraldehyde with subsequent osmium tetroxide/RR mixture and embedding into

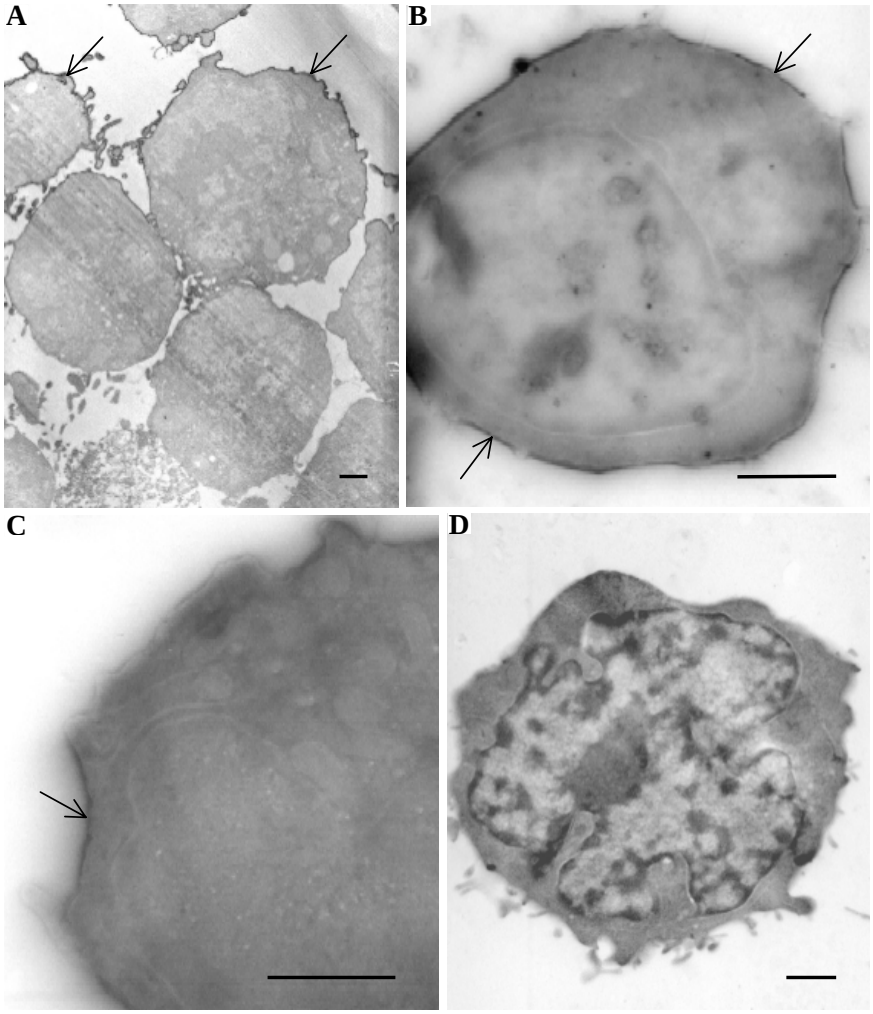

Figure 1. L1210 mouse leukemic cells. Arrows point to the surface coat stained by RR. **A**. Limited penetration of RR into the cell sediment following conventional double fixation and RR staining using glutaraldehyde with subsequent osmium tetroxide/RR mixture and embedding into Durcupan. **B**, **C**. Frozen sections after treatment with OsO_4 and RR. Mitochondria are negatively contrasted, the external coat has a positive contrast. **D**. Frozen section of a cell after treatment with uranyl acetate. Mitochondria are negatively contrasted, the external coat is not stained. Magnification bars: 1 μm.

Durcupan. The cells are linked together and RR staining (arrows) is limited only to superficial layer of the sediment. Towards the interior of the sediment the staining fades away.

On the other hand, the OsO_4 fixation and RR staining of cryosectioned cells by direct placing on the drop of solution enabled a simultaneous exposure of all cellular compartments to staining agents bypassing all diffusion barriers. Regardless of the position of a cell in the sectioned material, all structures were stained. The staining was a combination of a negative contrast (the plasma membrane and membranes of intracellular organelles) and of a positive contrast (cytoplasmic matrix and the extracellular coat) (Figs. 1B,C). The positive contrast of the extracellular coat containing glycoproteins, glycolipids and proteoglycans may be especially useful in studies comparing this external lamina in different cell populations.

The positive contrast of the extracellular coat did not appear after conventional and widely used method of negative contrasting with uranyl acetate as demonstrated in Fig. 1D.

Discussion

Ruthenium red staining of negative binding sites on cryosections of glutaraldehyde-fixed frozen cells has proved useful for uniform exposure of all cells and cellular compartments to the dye and especially of external coat containing glycoconjugates.

Handley and Chien (1981) have improved penetration of RR into intercellular regions by oxidizing RR with osmium tetroxide which resulted in reduction of cationic sites. However, the staining intensity and the thickness of the stained surface layer decreased.

The use of cryosections may be advantageous also in studies combining histochemical staining methods with immunocytochemical markers due to better preservation of antigens by cryofixation.

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