Lowicryl K4M Embedding of Brain Tissue for Immunogold Electron Microscopy

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We present methods for embedding brain tissue in Lowicryl K4M embedding medium and localizing antigens using postembedding immunogold techniques. After perfusion fixation with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M cacodylate buffer, blocks of rat brain were placed in 2% aqueous uranyl acetate for 1 hour, dehydrated in 50%, 70%, and 95% ethanol, infiltrated with Lowicryl/ethanol mixtures (1:2 for 10 min, 1:1 for 15 min) and 100% Lowicryl (20 min and 25 min). Polymerization was carried out under UV light for 24-48 hours at room temperature. Several neural antigens, including three different synaptic vesicle proteins and an enzyme associated with the postsynaptic density, were localized by this technique, indicating that this procedure may have wide applicability.

KEY WORDS: Lowicryl embedding methods; Neural antigens; Immunogold methods; Rodent brain.

Introduction

The introduction of water-miscible, low-temperature embedding media and the availability of good-quality colloidal-gold-conjugated second antibodies have made postembedding immunocytochemical methods for localization of antigens a reasonable alternative to preembedding immunoperoxidase methods (Roth et al., 1981; Carllemalm et al., 1982; Newman et al., 1983). Postembedding methods that do not require etching of plastic offer many advantages over preembedding methods. They are more rapid, penetration of antibody into the tissue is less of a problem, the same block of tissue can be used for many different antibodies, and double-labeling experiments are easier to perform. These methods have generally not been applied to studies of the brain, although kidney, retina, and pancreas have been amenable to study using published methods (Roth et al., 1981; Altman et al., 1984). The absence of evidence from brain may be due to the high lipid content of many areas of the brain and the fact that osmium has generally been avoided with these methods. We report here detailed methods for ultrastructural immunogold localization of neural antigens in brain using Lowicryl as an embedding medium, with preservation of both antigenicity and good-quality ultrastructure.

Materials and Methods

Rats were anesthetized with Nembutal and perfused through the heart with 100 ml saline (0.9%) followed by 250 ml of 4% paraformaldehyde, 0.1% glutaraldehyde, and 0.2 mM CaCl₂, in 0.1 M Na cacodylate buffer, pH 7.4. Brains were removed after 2 hr and placed in the same fixative without glutaraldehyde overnight at 4°C. Blocks approximately 1.0 mm³ in size were dissected out and placed in 0.1 M Na cacodylate buffer for 1 hr, with three changes. For some blocks, the aldehydes were quenched with 50 mM NH₄Cl in 150 mM NaCl, 100 mM NaPO₄₃, (phosphate-buffered saline, PBS, pH 7.4) (Brown and Farquhar, 1984). The blocks were then transferred to 2% aqueous uranyl acetate for 1-2 hr at 4°C in the dark. Dehydration was for 10 min each in 50%, 70%, and 95% ethanol (EtOH) or dimethylformamide (DMF) on ice. Lowicryl K4M (Polysciences, Warrington, PA) was mixed according to the following recipe: 2.7 g cross-linker A, 17.3 g monomer B, and 0.1 g bezoin ethyl ether, mixed under a stream of nitrogen (oxygen would inhibit polymerization). The tissue was then infiltrated with Lowicryl in the following steps: 2 parts absolute EtOH or DMF to 1 part Lowicryl for 10 min, 1 part EtOH or DMF to 1 part Lowicryl for 15 min, 100% Lowicryl for 20 min, and 100% Lowicryl for 25 min, all at room temperature using a rotator (Altman et al., 1984). The use of ethanol or dimethylformamide as a dehydration agent did not change the ultrastructural preservation.

The tissue was placed into BEEM capsules, which were filled with fresh Lowicryl and capped. Initial polymerization was for 45 min at 4°C, using a UV light source (longwave, 400 µW/cm², VWR Scientific) 12 inches from the blocks. UV polymerization was then continued for 24-48 hr at room temperature. In subsequent experiments, the entire polymerization was carried out at room temperature, with no adverse effects. BEEM capsules were suspended over the UV source, in a cardboard box lined with aluminum foil.

Semithin and ultrathin (silver-gold) sections were collected immediately after sectioning on glass slides or Formvar (1.0%) coated nickel grids (mesh or slot). Semithin sections were used for tissue identification and localization of particular areas of interest. Ultrathin sections of striatum, cerebellar cortex, and cerebral cortex were ex-
amine for localization of antibodies to synapsin I (De Camilli et al., 1983a, b), calcineurin (Klee et al., 1979), synaptic vesicle protein p65 (Matthew et al., 1981), and glycoprotein SV 2 (Buckley and Kelly, 1985). Optimum dilutions varied for each of the antibodies, and are given in the figure legends. Staining time in the first antibody was usually 5 min. Sections were treated with blocking antibody—5% normal goat serum (NGS) in PBS—prior to staining, with three 1 min washes. The 5% NGS–PBS was also used for dilution of antibodies and for washing grids. Grids were floated on drops for the antibody incubation and washing steps. Second antibodies were 20 nm gold goat antirabbit IgG and 10 nm gold goat antimouse IgG (Jannsen Pharmaceutica, Structure Probe Inc., West Chester, PA). Antibodies were used according to the supplier’s directions, either undiluted or at dilutions up to 1:10. Incubations were for 1 min. The grids were then carefully washed in NGS–PBS, followed by washing in double-distilled water. Controls were preimmune sera, monoclonal antibodies to irrelevant proteins, and second antibody alone.

Grids were stained in 2% aqueous OsO₄ for 10 min and in 2% aqueous uranyl acetate for 5 min and examined in a JEOL 100B electron microscope.

Results

Initial preparations used the method of Altman et al. (1984) as published. Briefly, this method consisted of immersion fixation in buffered 3% glutaraldehyde and 3% paraformaldehyde, followed by rapid dehydration in dimethylformamide, infiltration in Lowicryl, and 45 min UV polymerization. The tissue contained many holes and little ultrastructural detail could be observed (Figure 1A). Antibody binding was minimal (Figure 1B).

In order to improve the ultrastructure and increase antibody binding, we changed the following conditions: fixation, postfixation, heavy metal staining, length of polymerization, and antibody incubation times.

Fixation

Perfusion fixation with 4% paraformaldehyde and 0.1% glutaraldehyde has been used with success for preembedding peroxidase immunocytochemistry (Matthew et al., 1981), and even though the amount of glutaraldehyde was reduced from 3% to 0.1%, ultrastructural quality was improved (Figure 2). The reduction in glutaraldehyde also increased antigenicity (compare Figure 1B and 2B).

Figure 1. Electron micrographs of rat striatum, fixed and embedded according to the procedure of Altman et al. (1984). (A) Low power micrograph, showing holes throughout tissue. Area at left was myelinated fiber tract of internal capsule, which has not been preserved. (B) Section from same block incubated with supernatant against glycoprotein SV 2 for 5 min, and 10 nm gold goat antimouse IgG for 2 min (both undiluted). Arrows indicate synapse-like structure that is unlabelled. Stained with uranyl acetate and lead citrate. Original magnification: (A) × 15,000; (B) × 48,000. Bars = 0.1 micron.

Postfixation

Osmium tetroxide blocks the UV polymerization of Lowicryl (Carlsmalm et al., 1982). Therefore, uranyl acetate was chosen instead of osmium to increase contrast and stabilize lipids (Hayat, 1970, 1981). Tissue was incubated with 2% aqueous uranyl acetate for 1–2 hr at 4°C. The ultrastructural quality was greatly improved and antibody binding levels were about the same (compare Figure 2A and 2B, 2C and 2D).

Heavy Metal Staining

Original staining was with uranyl acetate and lead citrate. However, lead citrate deposited a precipitate on the tissue en bloc stained with uranyl acetate (not shown). This precipitate was not observed in the tissue prepared according to the Altman method (Figure 1). Staining with 2% aqueous osmium tetroxide and 2% aqueous uranyl acetate (after antibody binding) was found to produce the best contrast (Figures 2 and 3).

Polymerization

Holes in the tissue were eliminated by increasing the UV polymerization time to 24–48 hr.

Antibody Incubation Times

Original incubation times were for several hours or overnight, using dilute antibody solutions. While specific antibody binding could be observed, many of the membranes appeared to have been extracted. This was especially true for tissue that had not been treated with uranyl acetate (compare Figure 2A and B). Antibody binding times were greatly reduced (5 min) and antibody concentrations increased to give optimum staining. Even with short incubation times, tissue en bloc stained with uranyl acetate was preserved distinctly better than tissue without stain (compare Figure 2C with 2D, E, F).

Antibodies Used

This method was successful for a variety of antibodies. Antibodies to three synaptic vesicle protein antigens were localized with this technique: synapsin I (De Camilli et al., 1983a & b), p65 protein (Matthew et al., 1981), and glycoprotein SV 2 (Buckley and Kelly, 1985). These
proteins are probably present in different concentrations in vesicles, and staining with the antibodies was not uniform (Figure 2). Control sections showed very little or no gold labeling (Figure 2F). Another antibody used was to calcineurin, a $\text{Ca}^{2+}$-calmodulin dependent protein phosphatase present in high levels in the striatum (Wallace et al., 1980). Anti-calcineurin binding to Lowicryl sections of striatum showed that calcineurin appeared to be restricted to a subpopulation of cells and synapses in the striatum. Short incubations with the antibody showed specific labeling in the perikarya and proximal dendrites of some neurons (Figure 3A), including some dendrites and postsynaptic densities (Figure 3B).

Discussion

We have slightly modified the Lowicryl procedure of Altman (1984) in order to obtain good ultrastructure for immunocytochemical studies of the brain. The brain presents some special difficulties for electron microscopic studies, because immersion fixation is usually suboptimal and the high lipid content usually requires osmium in order to preserve myelinated fiber tracts. We were able to improve the ultrastructure by perfusion fixation, with a lower concentration of glutaraldehyde to conserve antigenicity, and by substituting uranyl acetate for osmium during postfixation. Osmium, which is usually used to fix lipid (Hayat,
Figure 3. A. Section of striatum incubated with antiserum against calcineurin (1:10) for 5 min, followed by 20 nm gold goat-anti-rabbit IgG (1:2) for 1 min. Labeling is confined to perikarya and proximal dendrites of some neurons. Arrows indicate aggregates of gold particles in proximal dendrite. Individual gold particles can also be seen. Nucleus is unlabeled. Bar equals 0.5 micron. B. Section of striatum incubated with antibodies as in A. Label can be seen in the postsynaptic density and throughout the dendrite. Osmium tetroxide and uranyl acetate stain. Original magnification (A) x 18,000; (B) x 48,000. Bar = 0.1 micron.

1970), cannot be used prior to UV polymerization of Lowicryl because it is UV opaque. Uranyl acetate can be used in this capacity, although not as efficacious as osmium, it does improve ultrastructure and retain antigenicity. The other factors important for improving the ultrastructure were to increase polymerization time and decrease antibody binding time.

We found, in agreement with Altman, that low-temperature embedding did not seem to be necessary for our antigens. Similarly, quenching of aldehydes did not produce greater antibody binding; but both of these conditions may need to be changed for some antigens.

The use of Lowicryl and immunogold techniques should be widely applicable to studies of nervous system antigens. Three different synaptic vesicle antigens and an enzyme associated with the postsynaptic density were visualized using these methods, demonstrating that this procedure should retain antigenicity for many protein antigens. The localization of these antigens was similar to that seen with immunoperoxidase methods (Buckley and Kelly, 1985; DeCamilli et al., 1983a and b; Matthew et al., 1981; Wood et al., 1980). For the synaptic vesicle antigens, the amount of label was less than that seen with immunoperoxidase. This was probably because only a few antigenic sites were exposed per synapse in a given thin section. For antigens that are present in greater quantity, such as neurotransmitters, sensitivity of the method should present no problem.

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Literature Cited


