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Technical Note

Microwave Fixation Improves Antigenicity of Glutaraldehyde-sensitive Antigens While Preserving Ultrastructural Detail

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Microwave fixation for electron microscopy has been used primarily for post-embedding immunocytochemistry. The present study examined the ability of microwave fixation to preserve the antigenicity of glutaraldehyde-sensitive antigens for pre-embedding immunocytochemistry. Five monoclonal antibodies (MAbs) directed against cell surface components of rat mast cells were tested. The MAbs failed to show any labeling of conventionally fixed rat bone marrow-derived mast cells even at glutaraldehyde concentrations as low as 0.1%. Strong staining of mast cell plasma membranes was seen when bone marrow was initially fixed with 2% formaldehyde and then refixed in 2% glutaraldehyde/2% formaldehyde after immunostaining. However, the ultrastructural preservation of the cells was poor. Antigenicity and morphological detail were both preserved when bone marrow was fixed in 0.05% glutaraldehyde/2% formaldehyde for 4 sec in a 550-W microwave oven. With this method, mast cells in various stages of maturation as well as cells that did not contain granules were immunoreactive. This method should prove useful with antigens from many different cell types that are sensitive to glutaraldehyde fixation. (*J Histochem Cytochem 43:307-311, 1995*)

KEY WORDS: Microwave fixation; Electron microscopy; Immunocytochemsitry; Glutaraldehyde-sensitive antigens; Mast cells.

Introduction

Since its introduction by Mayers in 1970 (24), microwave irradiation has been explored as a fixation method for a wide variety of light and electron microscopic techniques (10,15) including routine histology (2,5,8,14,16,18,22,24,29), electron microscopy (3,8, 13,17,18,23), enzyme cytochemistry (25) and immunolabeling (8, 13,15-17,19,20,21,23,26,27). Although the exact mechanism of microwave fixation is unknown, it is thought to lie primarily in the heat generated by increased molecular movement (12). Microwaves consist of an alternating electromagnetic field that changes direction 2,450,000,000 times every second. Water and other dipolar molecules in the sample oscillate at this frequency, which increases their thermal agitation and generates heat. The molecular movement may also aid the penetration of chemicals into tissues and cells. The major advantage of microwave fixation has been its speed, seconds or minutes compared to hours for conventional chemical fixation. Initially microwave fixation was performed on small blocks of tissue in air. Gordon and Daniel (5) showed that this produced hardening and drying of the blocks and suggested that fixation for light microscopy be done in water, saline, or formalin. For electron microscopy, Login and Dvorak (18) introduced the use of a dilute Karnovsky's fixative (9) combined with microwave irradiation. This method gave excellent morphological preservation and has been successfully used for immunolabeling for both light and electron microscopy (15,17,19,20). Because very low concentrations of glutaraldehyde are employed, this method should be ideal for electron microscopic immunocytochemistry of sensitive antigens. The present study extends the use of microwave fixation in a dilute aldehyde solution to detect glutaraldehyde-sensitive antigens on the surface of rat bone marrow-derived mast cells while at the same time retaining ultrastructural detail.

Materials and Methods

Cells. Bone marrow from the femurs of young (150 g) male and female Wistar rats was removed with Dulbecco's PBS containing heparin (1000 U/ml) (Produtos Roche Quimicas e Farmaceuticos SA; Rio de Janeiro, Brazil). The cells were dissociated by aspiration with a Pasteur pipette. They were then rinsed twice by centrifugation (27 × g) in PBS and fixed immediately.

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Microwave Calibration. The procedure used was a modification of one described by Login and Dvorak (16). A conventional microwave oven (Montgomery Ward Model 8035) with a maximal power output of 550 W and an operating frequency of 2450 mHz was used without modification. A glass beaker containing 100 ml of distilled water was placed in the right rear corner of the unit. The water load was pre-heated for 2 min to warm up the magnetron. The water load was replaced with 100 ml distilled water (25°C). Two percent Giemsa/agar blocks (0.5 cm^3) were made by completely dissolving the electrophoresis-grade agar (North–Strong; Rockville, MD) in 0.9% saline solution and adding Giemsa solution (Sigma Chemicals; St Louis, MO) to the liquid agar to a final concentration of 0.5% (15). The Giemsa/agar solution was poured into flat embedding molds and allowed to solidify. The resulting blocks were then trimmed into cubes or used as they came from the molds. The Giemsa/agar blocks were immersed in 5 ml of fixative (0.05% glutaraldehyde) (Ladd Research Industries; Burlington, VT), 2% formaldehyde (Ladd), 0.025% $CaCl_2$ in 0.1 M cacodylate buffer, pH 7.4, in 35-mm plastic tissue culture dishes (one block per dish) located at various positions on the floor of the microwave unit and exposed to microwave radiation at 100% power. The color change of the Giemsa/agar blocks served as a monitor for temperature change during fixation. By placing the blocks in various locations on the floor of the oven and then irradiating them, the optimal position of the sample during microwave fixation could be determined.

Microwave Fixation. Cells were suspended in 5 ml of fixative in 35-mm plastic tissue culture dishes and irradiated for 4 sec at 100% power. Immediately after irradiation, the cell suspension was added to 10 ml PBS and centrifuged for 2 min at 27 × *g*. The cells were then rinsed twice in PBS, PBS containing 0.1 M glycine, and then in PBS.

Immersion Fixation. Cells were suspended in 5 ml of fixative containing 0.1% glutaraldehyde (Ladd), 2% formaldehyde (Ladd) in 0.1 M cacodylate buffer (pH 7.4) or in 2% formaldehyde in 0.1 M cacodylate buffer (pH 7.4) for 30 min at room temperature (RT). Then 10 ml of PBS was added to the suspension and the cells processed as above.

Antibodies. Monoclonal antibodies (MAbs) were raised in mice against the cell surface of RBL-2H3 cells, a rat mast cell line. MAb AA4 (6) recognizes novel gangliosides on rat mast cells, MAb BC4 is directed against the α-subunit of the high-affinity IgE receptor (1), AD1 (11), BD1, and BGD6 (7) are all against cell surface proteins on rat mast cells.

Immunostaining. After fixation, cells were incubated with primary antibody (MAb AA4, 2.5 μg/ml; MAb BC4, 0.5 μg/ml; MAbs AD1, BD1, and BGD6; 10 μg/ml) diluted in PBS + 1% BSA for 1 hr at RT. After incubation the cells were rinsed sequentially in PBS + 1% BSA, PBS, PBS + 1% BSA and then incubated for 1 hr with goat anti-mouse IgG conjugated to horseradish peroxidase (25 μg/ml final concentration) (Jackson ImmunoResearch; Avondale, PA) diluted in PBS + 1% BSA. Cells incubated without primary antibody served as controls. The cells were then rinsed in PBS + 1% BSA, PBS, and 0.1 M cacodylate buffer (pH 7.4) and immersed in diaminobenzidine (DAB) incubation medium (12.5 ml cacodylate buffer, 12.5 mg DAB) (Polysciences; Warrington, PA) and 250 μl 1% H_2O_2 for 30 min at RT. The cells were then rinsed 10 times in cacodylate buffer. Some samples were packed by centrifugation in 2% agar. Cells were suspended in 2% agar at 60–65°C and centrifuged at 127 × g for 5 min.

Sample Processing. Cells were post-fixed in 2% OsO_4 (EM Sciences; Ft Washington, PA) for 1 hr at RT, rinsed in distilled water, dehydrated through a graded series of ethanols, rinsed in acetone, and embedded in Embed 812 (EM Sciences). Thin sections were cut with a diamond knife and stained for 10 min each in Reynolds' lead citrate (28) and uranyl acetate.

Results

Microwave fixation in dilute aldehydes proved to be an excellent method to preserve both morphological detail and antigenicity of rat bone marrow-derived mast cells. Calibration of the microwave oven was critical to the success of this method. Ideal positioning of the samples and time of microwave irradiation were based on the following criteria: (a) a uniform color change in the agar block; (b) a rapid rise in the temperature of the fixative in the dishes (to 50°C in 10 sec or less); and (c) an increase in the temperature of the water load to 30–35°C. Uniform color change of the agar blocks from violet to dark blue without any melting of the agar proved to be the most accurate indicator of proper fixation conditions. At RT the Giemsa/agar blocks are violet, between 40°C and 50°C they are dark blue, and between 55°C and 60°C they become light blue. The color change from violet to dark blue is an accurate monitor of sample temperature during the fixation procedure.

Cells fixed with the microwave irradiation protocol showed excellent morphology (Figures 1a, 1d, and 2a–2d). Cell organelles, including the nucleus, secretory granules, mitochondria, and Golgi apparatus were all well preserved. Most importantly, the antigenicity of plasma membrane components (Figures 1d and 2a–2d) was retained. This was true for both lipid and protein constituents of the plasma membrane. Control cells that were fixed by microwave irradiation but immunostained without primary antibody showed good ultrastructural preservation, but no reaction product was present on the plasma membrane of any cells in the sample (Figure 1a). Antigenicity could be preserved by fixing the cells in formaldehyde without glutaraldehyde, but in this instance cellular fine structure was not preserved (Figure 1b). In contrast, when the cells were fixed conventionally by immersion fixation, the levels of glutaraldehyde required to preserve the integrity of the cells destroyed the antigenicity of the membrane components toward the antibodies tested (Figure 1c).

Discussion

Microwave fixation provides an excellent method of preserving both ultrastructural detail and antigenicity, especially of glutaraldehyde-sensitive antigens. The ability of glutaraldehyde to cross-link proteins through their amino groups and thus preserve fine structural details has made it an ideal fixative for electron microscopy. However, the use of glutaraldehyde during fixation can have a major impact on the ability to immunolabel a specimen (4). This can be a particular problem when MAbs are used that are specific for only one epitope. The cross-linking of proteins by glutaraldehyde may change their conformation, or the presence of glutaraldehyde may limit the accessibility of the antibody to its antigen. Microwave fixation permits the use of extremely low concentrations of glutaraldehyde while still yielding excellent ultrastructural detail. These low concentrations of glutaraldehyde do not appear to interfere with the ability to immunolabel the surface of mast cells with a variety of MAbs. The antigenicity of both membrane-associated proteins and glycolipids was well maintained. The results presented here demonstrate that microwave fixation is well suited to pre-embedding immunocytochemistry.

Microwave fixation has been used successfully for post-embedding immunostaining for both light and electron microscopy. For light microscopy, fixation has been by microwave irradiation in PBS (8,13) or by microwave irradiation in an aldehyde solution (15,20).

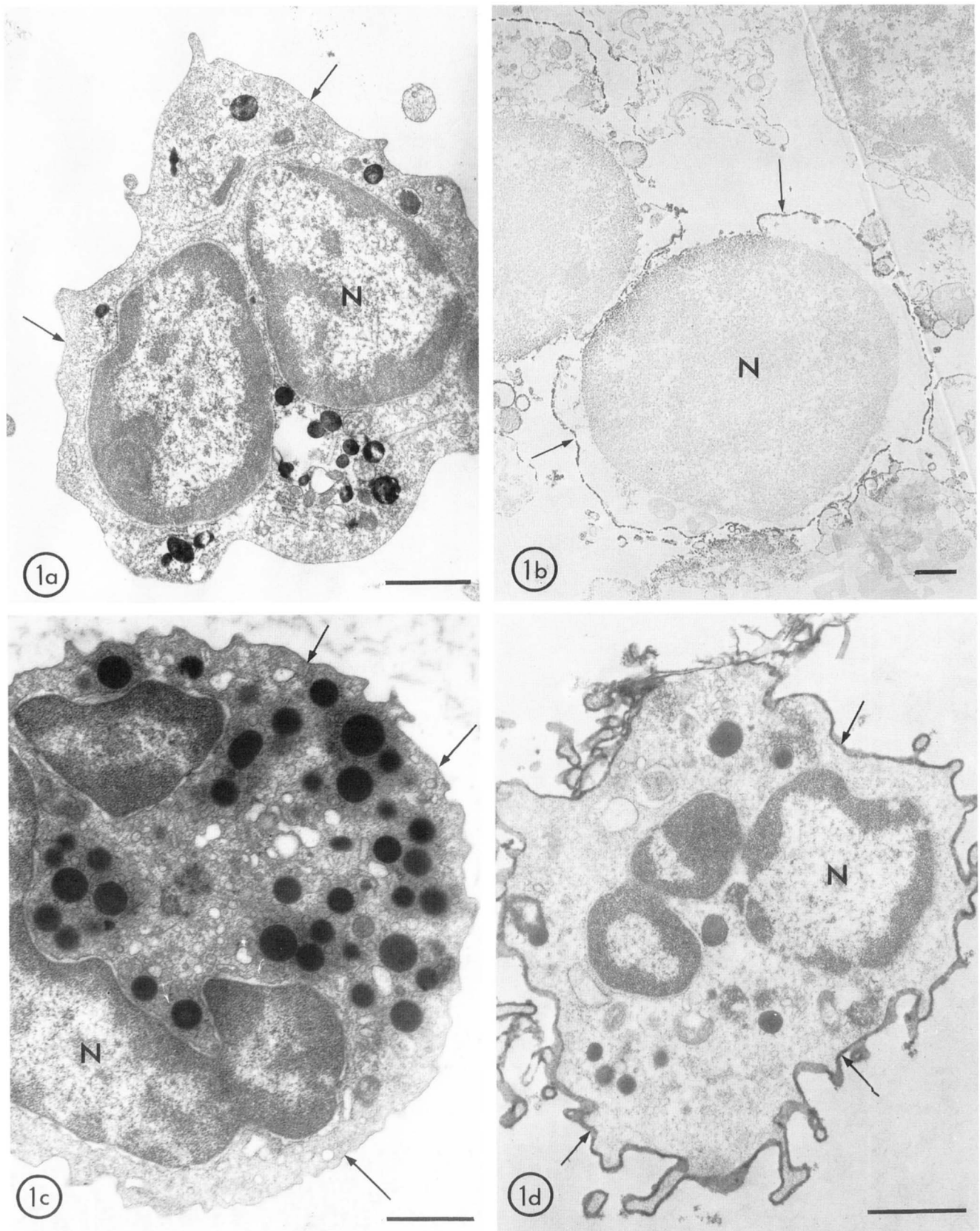

Figure 1. Rat bone marrow-derived mast cells. (**a**) Microwave fixation; no primary antibody. Microwave fixation gives excellent morphology. There is no staining of the plasma membrane (arrows) when the primary antibody is omitted from the procedure. N, nucleus. (**b**) Formaldehyde fixation; immunostained with MAb AA4. Although the plasma membrane is well stained (arrows), little morphological detail is preserved. (**c**) Glutaraldehyde fixation; immunostained with MAb AA4. Although the cell is well fixed, there is no staining of the plasma membrane (arrows). (**d**) Microwave fixation; immunostained with MAb AA4. There is excellent preservation of cellular detail and staining of the plasma membrane (arrows). Original magnifications: **a** × 14,000; **b** × 8000; **c** × 17,000; **d** × 18,000. Bar = 1 μm.

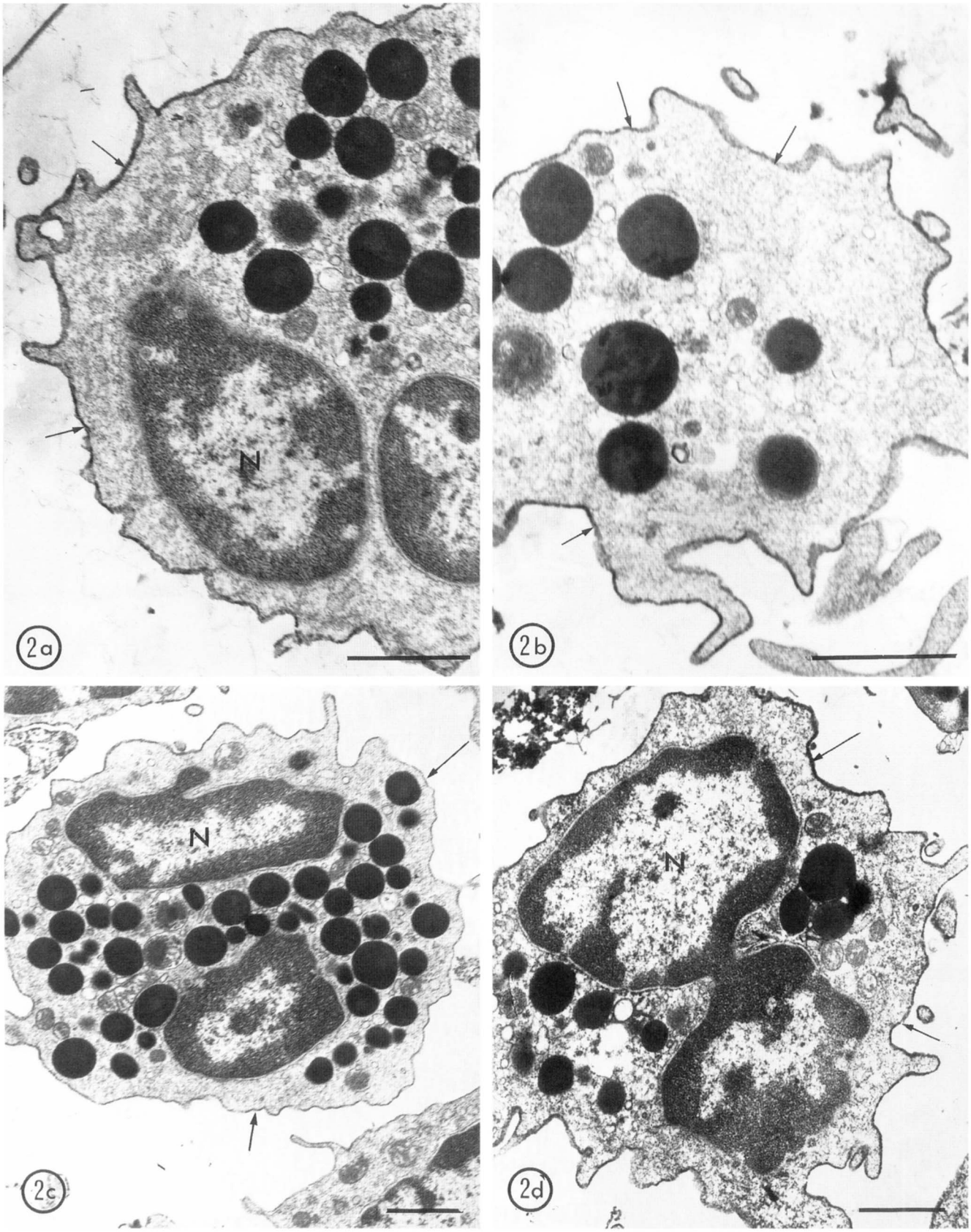

Figure 2. Rat bone marrow-derived mast cells, microwave fixed. The morphological preservation was excellent and all four antibodies tested, (**a**) MAb AD1, (**b**) MAb BC4, (**c**) MAb BD1, and (**d**) MAb B_GD6 showed extensive staining of the plasma membrane (arrows) of bone marrow-derived mast cells. N, nucleus. Original magnifications: **a** × 23,000; **b** × 26,000; **c** × 13,000; **d** × 18,000. Bars = 1 μm.

Microwave fixation, alone or combined with aldehydes, gave excellent preservation of morphological detail and antigenicity was retained in a wide variety of tissues. In addition, when specimens were fixed by microwave irradiation in aldehydes it was possible to reduce the concentration of the primary antibodies by tenfold compared with conventionally fixed material and still to achieve excellent immunostaining. Moreover, it was not possible to stain for keratin in the epithelium of formalin-fixed skin, although specimens fixed by microwave irradiation stained well (17). For light microscopy, microwave fixation has been shown to have an advantage over conventional fixation for immunohistochemistry.

For electron microscopic immunocytochemistry, microwave fixation has been used primarily for post-embedding immunostaining of mast cells (15,19,20). The method has been applied preferentially to mast cells because they degranulate easily and are difficult to preserve. After microwave fixation, both chymase (20) and histamine (19) have been immunolocalized in rat peritoneal mast cell secretory granules. However, the microwave fixation did not appear to offer any advantage over conventional fixation in preserving antigenicity. This is in contrast to the present results, in which microwave fixation was clearly superior in preserving the antigenicity of known glutaraldehyde-sensitive antigens. Preliminary studies in our laboratory indicate that microwave fixation will also be useful for demonstrating glutaraldehyde-sensitive antigens intracellularly. The same panel of antibodies employed in this investigation has been used successfully to localize antigens intracellularly after microwave fixation in a cultured rat mast cell line, RBL-2H3 cells. Microwave fixation should be useful in preserving antigenicity of glutaraldehyde sensitive antigens in general and should have wide applicability to other cells and tissues.

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