Recovery of Nuclei From Glycol-Methacrylate-Embedded Tissue

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The analysis of antigens, enzyme histochemical markers, and DNA has become an important part of the classification of some leukemias, lymphomas, and other neoplastic diseases. Many of the relevant antigens and most of the relevant enzyme histochemical activities are destroyed and others are less than optimally preserved in tissues embedded in hot paraffin. Most enzymatic activities and antigens are well preserved in tissues embedded at 4°C in glycol methacrylate (GMA). The measurement of DNA content in neoplastic cells with the most commonly employed techniques depended on the availability of fresh suspensions of cells until the development by Hedley of methods that permit the recovery of nuclei from paraffin blocks for this purpose. In order to facilitate the analysis of antigens, enzymatic markers, and DNA from the same sample of tissue, we have developed a means of recovery of nuclei from GMA-embedded tissues. Twenty-μm-thick sections of GMA-embedded tonsil were either pretreated with an organic solvent (absolute ethanol or 2-ethoxyethanol) followed by rehydration in phosphate buffered saline (PBS) or directly rehydrated in PBS. The suspensions were formed mechanically by gentle sonication. The type of fixative and length of PBS rehydration were varied. Tissue fixed in 100% acetone, embedded in GMA, and rehydrated directly in PBS for six days gave the highest average yield of nuclei, 3.7 x 10^7 nuclei per gram tissue. In order to assess DNA binding of fluorescent dyes, 2-μm-thick GMA sections were stained with chromomycin, Hoechst 33342 (Sigma Chemical, St Louis, MO), and propidium iodide, Hoechst 33342 bound specifically to the nuclei with low background staining. © 1990 by The American Society of Hematology.

F LOW CYTOMETRY, immunohistochemistry, and enzyme histochemistry are useful techniques for evaluation of cell proliferation and transformation.1-5 The demonstration of many antigenic and enzymatic activities that are not optimally demonstrated in paraffin-embedded tissues has been useful for the investigation of normal elements of the blood and marrow6 and for the classification of leukemia and lymphoma.7,8 The demonstration of many of these activities in glycol methacrylate (GMA) has been described, and the diagnostic application of these methods for the characterization of bone marrow biopsies has been reviewed.9 Populations of cells can be fractionated and quantified with flow cytometry, whereas the structural and functional properties of cells within tissues can be studied with immunohistochemistry and histochemistry. Analysis of paraffin-embedded tissues by flow cytometry makes possible both prospective and retrospective studies of many human malignancies by simultaneously assessing DNA content and histological parameters for correlation with prognosis.10-12 The expression of some nuclear and nucleolar antigens has been reported to be altered in neoplastic diseases and has, in some cases, been related to both staging and prognosis.13-17 However, the high temperatures involved in paraffin embedding can denature nuclear antigens associated with cell proliferation or neoplasia.4

Because low temperatures are used in the GMA-embedding technique, antigen structure and enzyme activity are preserved for immunohistochemistry and enzyme histochemistry.1,3,4,8-10 Fixatives such as acetone, when substituted for formalin, result in better preservation of some antigens.1 The preservation of some nuclear and nucleolar antigens after being embedded in GMA at 4°C may facilitate the investigation of the expression of these antigens by flow cytometry. We describe a method to obtain nuclei from GMA-embedded tissue, a prerequisite for the analysis of these tissues by flow cytometry.

MATERIALS AND METHODS

Tissue. Eight tonsils were obtained at surgery by the Tissue Conservation Core Facility of the Case Western Reserve University Cancer Center after approval was obtained for these studies from our Institutional Review Board and in accordance with an assurance filed with, and approved by, the Department of Health and Human Services. The tonsils were frozen over liquid nitrogen as 1-mm-thick slices of tissue. Four samples from each patient were weighed and embedded in GMA: two pieces were fixed in 2% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.4, for 2 hours at 4°C; and two pieces were fixed in 100% acetone overnight at -20°C.

Embedding. Tissue was embedded in GMA as described.3 Fixed tissue was rinsed with 0.1 mol/L phosphate buffer (pH 7.4); soaked for 3 hours in 0.1 mol/L phosphate buffer (pH 7.4) with 3% sucrose; dehydrated under vacuum of 17 in of mercury in 50%, 75%, and 95% anhydrous methanol; and infiltrated with glycol methacrylate monomer (JB-4 solution A, Polysciences, Warrington, PA) under vacuum of 17 in of mercury overnight. The tissue was placed in embedding molds (BioRad Microscience Division, Cambridge, MA) and the molds were filled with embedding medium comprised of 20 mL JB-4 solution A, 0.09 g benzoyl peroxide, and 0.5 mL of JB-4 solution B. Blocks were stored at 4°C. Overnight acetone-fixed tissue was infiltrated directly with GMA monomer at the same time as the 2% paraformaldehyde-fixed tissue. All subsequent steps in embedding were identical for the two methods of fixation. The blocks were brought to room temperature and cut at 20-μm intervals on a Lipshaw 45 Rotary Microtome (Lipshaw Manufacturing, Detroit, MI) with a glass knife adapter (LKB, Rockville, MD). If necessary, the blocks could be softened by placing them in a humid chamber.
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All of the tissue was used in the experiment, i.e., approximately 100 20-μm-thick sections were divided evenly among four 15-mL glass centrifuge tubes one section at a time.

Preparation of nuclear suspensions. Nuclear suspensions were obtained from GMA-embedded tissue with the use of a TMS 40 sonicator (Tekmar, Cincinnati, OH). All procedures were carried out at room temperature unless otherwise specified. GMA sections in the first tube were pretreated overnight with 3.0 mL of absolute ethanol; those in the second tube with 3.0 mL of 2-ethoxyethanol (Aldrich Chemical Company, Milwaukee, WI); and those in the third tube with 3.0 mL of phosphate-buffered saline with 0.15 mol/L NaCl, pH 7.4 (PBS/NaCl; 8.0 g NaCl, 1.15 g Na₂HPO₄, 0.20 g KH₂PO₄ in 1000 mL distilled H₂O with 9.75g NaCl). After these solvents were decanted, the sections were washed with PBS, and then left standing in 3.0 mL PBS/NaCl, for the periods of time indicated below.

Incubation in PBS/NaCl was studied for six days in all eight patients and for three days in four patients. After incubation, the PBS was decanted, five drops of 0.5% Harris-modified hematoxylin (Fisher Diagnostics, Orangeburg, NY) were added, and the tissue was stained for five minutes and washed with PBS. The tissue from each tube was transferred to two 1.5-mL plastic centrifuge tubes (Sarstedt, Newton, NC) and suspended in 1.0 mL PBS for sonication. The sonicator was set for 20 watts output, and each sample was sonicated twice for 20 seconds with stirring in between sonications to unfold GMA sections. An ice-water bath was used to prevent overheating of the samples during sonication, and laboratory film was used to cover the samples to minimize losses from aerosolization. After sonication the nuclear suspension was filtered through 250-μm nylon mesh (Tetko, Elmsford, NY), and the remaining tissue was resuspended in fresh PBS. Intermediate nuclear yields were quantified with a hemocytometer (American Optical, Buffalo, NY). Sonication and quantification were repeated twice. Finally, suspensions from the three trials were pooled, filtered through 37-μm nylon mesh, and centrifuged for 10 minutes at 200g. The excess supernatant was decanted, and the overall yield was calculated.

Fluorescence microscopy. Three fluorescent dyes, propidium iodide, chromomycin, and Hoechst 33342 (all from Sigma Chemical Company, St Louis, MO), were used to visualize nuclei with a fluorescence microscope (Nikon, Garden City, NY). Each dye was made up in a solution of PBS containing 1% (vol/vol) Triton-X-100 (Sigma Chemical Company, St Louis, MO). Propidium iodide concentration was 50 μg/mL, chromomycin concentration was 100 μg/mL, and Hoechst 33342 concentration was 1.0 μg/mL. The chromomycin solution also contained 75 mmol/L MgCl₂. Two-μm-thick GMA sections were incubated in each dye for 10 minutes.

RESULTS

When viewed in the hemocytometer, the suspensions obtained after sonication and filtration were mononuclear. Intracellular detail, such as nuclei, was plainly visible in hematoxylin-stained nuclei as shown in Fig 1. Lymphocyte nuclei were the predominant nuclear type, but occasional larger nuclei with diffuse chromatin and prominent nucleoli were visible. A thin rim of GMA was seen investing each nucleus.

Nuclear yields are summarized in Table 1 and Fig 2. In general, higher nuclear yields were obtained from tissue fixed in acetone than from tissue fixed in 2% paraformaldehyde. This difference between fixatives was significant (P < .05) for tissue soaked in PBS alone and for tissue pretreated with 2-ethoxyethanol followed by incubation in PBS; however, this difference did not reach significance (P < .10) for tissue pretreated with ethanol followed by incubation in PBS. Soaking in PBS for 6 days resulted in a slightly increased nuclear yield; however, the difference between 3- and 6-day PBS incubation was not significant. Likewise, higher nuclear yields were seen from tissues treated with PBS alone than from tissues pretreated with ethanol or 2-ethoxyethanol; however, the differences were not significant.

The GMA tissue sections stained with Hoechst 33342 had bright nuclear staining with little or no background fluorescence. Nuclear fluorescence was weaker with propidium...
iodide, and the dye also bound nonspecifically to the GMA. Chromomycin stained the nuclei weakly and also bound to collagen in the stroma.

**DISCUSSION**

Variables affecting nuclear yield can be divided into three categories: tissue preparation, treatment prior to sonication, and the experimental conditions used for the sonication.

Tissue preparation involved fixation, embedding, and sectioning. Because resting lymphocytes are 4 to 10 μm in diameter, 20-μm sections were used so that sections were 2 to 5 cells thick. Nuclear recovery occurred by mechanical action; nuclei closest to the surface were liberated, but deeper nuclei were trapped. With most blocks, 20-μm sections were easily cut if the sections were brought to room temperature for a few hours in a closed chamber. If necessary, blocks were softened by placing them in a humid chamber. When processing tissues with larger nuclei, especially tumor nuclei, thicker sections are desirable to avoid cutting across nuclei; 50-μm sections can be cut with this technique. The method of fixation seems to have the largest effect on nuclear yields. Baker classifies acetone as a nonadditive, coagulative fixative in contrast to formaldehyde which reacts with amino-terminal groups and is noncoagulative. It is possible that acetone coagulates cytosolic proteins and removes water, making the cytosol less permeable to GMA monomer, therefore interrupting polymerization.

Initially it was desired to clear the GMA from the tissue sections with an organic solvent prior to sonication. Absolute ethanol and 2-ethoxyethanol have been used to soften glycol methacrylate (verbal communication, several polymer chemists). Both absolute ethanol and 2-ethoxyethanol caused the GMA to swell without noticeable solvolysis. PBS caused swelling and softening of the gel and led to equivalent or higher yields than did pretreatment with ethanol or 2-ethoxyethanol.

The use of GMA as an embedding medium has been found advantageous for the demonstration of those antigens and enzymes that do not survive being embedded in hot paraffin. Some antigens in lymphoid tissue have shown enhanced preservation after acetone fixation. DNA analysis by flow cytometry has been shown to be related to prognosis in gastric, colon, prostate, pituitary, and breast neoplasms. In addition, simultaneous quantification...
of the expression of nuclear antigens and DNA content has lead to better understanding of the biology of proliferating cells.\textsuperscript{1,3,13,12} The recovery of whole nuclei from GMA sections will facilitate the exploitation of these advantages for the investigation of nuclear structures. Quantification of the expression of nuclear and nucleolar antigens with cytometric techniques in methacrylate may facilitate valuable clinical correlations.

**ACKNOWLEDGMENT**

We thank Tammy Fowler for her help in preparing this manuscript.

**REFERENCES**

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