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Rapid Communication

The AMeX Method: A Multipurpose Tissue-processing and Paraffin-embedding Method

II. Extraction of Spooled DNA and Its Application to Southern Blot Hybridization Analysis

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In our previous report, we described a new fixation and paraffin-embedding method (the AMeX method) that preserves many of the antigens that are normally destroyed by routine formalin fixation. The current study was conducted to examine the preservation of high-molecular-weight DNA in tissues processed by this method. DNA was extracted from AMeX-processed tissue sections after deparaffinization by the same method as that used to extract DNA from fresh tissues. The total amounts of DNA extracted from 10 mg each in wet weight of AMeX-processed and fresh mouse liver tissues were identical. In tissues of malignant lymphoma, the total amount of spooled DNA extracted from 50 sections, each 20 μm thick, was about 8 μg/mm². The electrophoretic pattern of DNA digested with restriction endonucleases on agarose gel from AMeX-processed tissue sections did not differ from that of fresh materials. Southern blot hybridization analysis also revealed that the mobility of specific DNA fragments was identical for AMeX-processed and fresh tissues. The AMeX method was thus proved to be a versatile multipurpose tissue-processing procedure, which is expected to provide important information regarding the correlation between morphology, phenotypic expression, and gene alteration. (Am J Pathol 1990, 136:267-271)

Recently two different methods for extraction of DNA from routinely processed formalin-fixed paraffin-embedded tissues were described.[1,2] Debeau et al[1] demonstrated that spooled DNA suitable for Southern blot hybridization analysis may be obtained from formalin-fixed paraffin-embedded tissue blocks. However, the extracted DNA was not intact and somewhat fragmented when compared with DNA extracted from fresh material. Because of this limitation, DNA extracted from formalin-fixed paraffin-embedded tissues has been analyzed most frequently by DNA-DNA dot- or slot-blot hybridization techniques.[3,4]

Recently we developed a simple method (the AMeX method) for better preservation of many antigens that cannot be detected in routinely fixed paraffin-embedded tissue sections.[5] In the present study, preservation of DNA in tissues processed by this method was examined to confirm its versatility as a multipurpose tissue-processing method suitable for morphologic, immunohistochemic, and molecular biologic analyses.

Materials and Methods

Twenty-five cases of malignant lymphoma were used in this study. They were divided into 10 T-cell lymphomas and 15 B-cell lymphomas by immunohistochemical staining for surface markers of T and B lymphocytes. Tissues were immediately fixed and processed by the AMeX method after excision.[5] Briefly, tissues were immersed in acetone at 4 C, transferred to a freezer at −20 C, and

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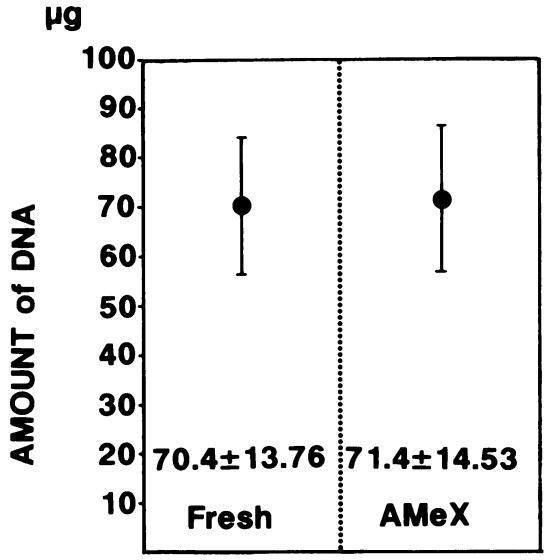

Figure 1. *DNA was extracted from AMeX-processed and fresh liver tissue, each sample 10 mg in wet weight, from three different portions of three different mice. Each value was given by mean ± standard deviation of nine samples each.*

fixed overnight. Then the tissues were dehydrated in acetone at 4 C for 15 minutes and in acetone for another 15 minutes at room temperature. Thereafter the tissues were cleared twice in methyl benzoate for 15 minutes, and twice in xylene for 15 minutes at room temperature. Finally they were penetrated with paraffin (melting point, 58 to 60 C; Junsei Chemical Co., Ltd., Tokyo, Japan) at 60 C for 2 to 3 hours in a vacuum-evaporating embedder and embedded in paraffin.

For DNA extraction, 50 sections, each 20 μm thick, were cut with a microtome and collected in a 50-ml centrifuge tube. Then they were deparaffinized by suspension in 20 ml of xylene for 5 to 10 minutes and washed by centrifugation in xylene, acetone, and 0.1 mol/L (molar) phosphate-buffered saline (PBS; pH 7.4), two times each. Finally the pellet was resuspended in 10 ml of lysis buffer consisting of 1% sodium dodecyl sulfate (SDS), 0.2 mol/L NaCl, 20 mmol/L Tris-HCl, pH 7.4, 2 mmol EDTA, and 100 μl of 10 mg/ml proteinase K (E. Merck, Darmstadt, Germany) and incubated overnight at 37 C. DNA was extracted twice with phenol/chloroform and twice with chloroform, and precipitated with three volumes of 100% ethanol containing 0.3 mol/L sodium chloride. Then DNA was spooled with a glass rod, washed with 70% ethanol, dissolved in 1× TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0), and stored at 4 C. For comparison, DNA extracted from fresh tissue of seven of the above cases was used as a control.

To assess the loss of DNA in AMeX-processed tissue, the amount of DNA extracted from 10 mg each in wet weight of AMeX-processed and fresh tissues of the liver of a Balb/c mouse was compared. This experiment was repeated nine times using three mouse livers.

EcoRI or BamHI restriction endonuclease-digested DNA fragments were electrophoresed on 0.8% agarose gel in 1× TAE buffer (40 mmol/L Tris-acetate, 1 mmol/L EDTA) with 50 μl of 20 mg/ml ethidium bromide. The electrophoretograms were then visualized by ultraviolet illumination. The DNA was then transferred to nitrocellulose filters (Schleicher & Schuell, Inc., Keene, NH) using the procedure of Southern.[6] Each filter was baked under vacuum for 2 hours at 80 C and hybridized with nick-translated ^{32}P-labeled probes. The probes used in this study were the 3.5 kilobase pair (kbp) EcoRI-HindIII fragment of the immunoglobulin heavy-chain joining region (JH), and the 0.77 kbp Pst I fragment of the T-cell receptor β-chain (TcRβ). The nitrocellulose filter was prehybridized for 3 to 4 hours at 42 C in a solution containing 50% formamide, 5× Denhardt's solution, 0.1 mol/L piperazine-N,N'-bis[2-ethanesulfonic acid](pH 6.8), 0.65 mol/L sodium chloride, 5 mmol/L EDTA (pH 7.5), 0.1% (SDS), and 100 μg/ml denatured salmon testis DNA (Sigma Chemical Co., St. Louis, MO). The prehybridization solution was then discarded, and ^{32}P-labeled probes in the same solution, along with 200 μg/ml denatured salmon testis DNA and 10% dextran sulfate were hybridized to the filter overnight at 42 C. After hybridization, the filter was washed twice for 15 minutes at room temperature in 0.5× SSC (75 mmol/L sodium chloride-7.5 mmol/L sodium citrate)-0.1% SDS, and then for 30 minutes at 55 C in 0.5× SSC-0.1% SDS. The filter was exposed to Kodak XAR-5 X-ray film with two intensifying screens overnight at −80 C.

Results and Discussion

Spooled high-molecular-weight DNA was recovered from AMeX-processed tissue sections in every case. The

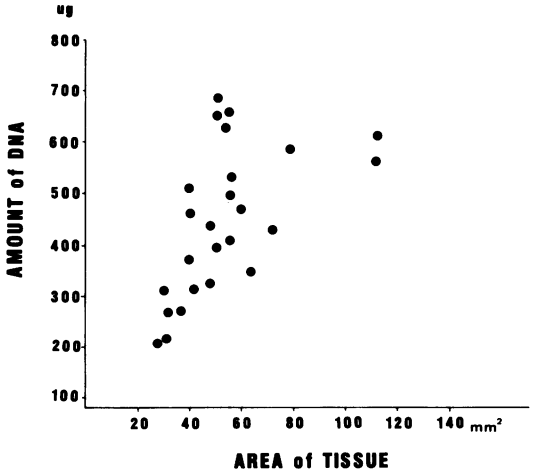

Figure 2. *Total amount of spooled high-molecular-weight DNA extracted from 50 sections, each 20 μm thick, in AMeX-processed tissue from malignant lymphoma cases. Abscissa indicates the area of the tissue block. Ordinate indicates the amount of extracted DNA. Total amount of DNA showed a linear correlation with the area of tissues of malignant lymphoma.*

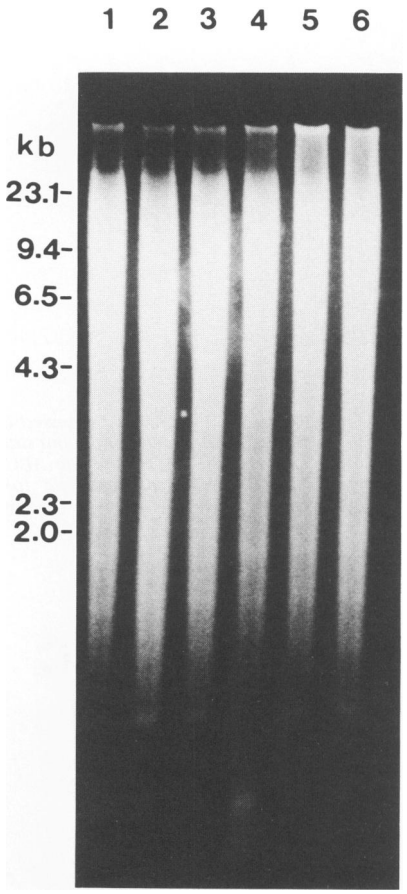

Figure 3. *Effect of storage in paraffin for different periods of time on the electrophoretic pattern of restriction DNA fragments. Lanes 1 to 6 represent different samples stored in paraffin at 4 C for 1, 6, and 12 months, and 2, 3, and 4 years, respectively. Ten micrograms of DNA was digested with BamHI, electrophoresed on agarose gel, and stained with ethidium bromide. Sizes of molecular-weight markers (lambda DNA cleaved with HindIII) are indicated in kilobase pairs (kb).*

method of DNA extraction from AMeX-processed tissue sections after the initial deparaffinization steps was not different from that used for fresh materials. Furthermore, spooled DNA recovered from AMeX-processed tissue blocks that had been kept at 4 C for more than 4 years did not show any decrease in the amount of high–molecular-weight DNA present. Recently Debeau et al[1] described a method for extraction of DNA suitable for Southern blot hybridization analysis from formalin-fixed paraffin-embedded tissue blocks, but the ratio of spooled DNA to total DNA was only 57%, even using tissues fixed for only 12 hours. These data were obtained from freshly fixed and embedded tissue blocks, so it is possible that the ratio of spooled DNA might have been decreased further in old paraffin blocks prepared for routine histopathologic examination.

The average amount of DNA extracted from AMeX-processed mouse liver tissues was 71.4 ± 14.53 μg per 10 mg in wet weight and that from fresh tissues was 70.4 ± 13.76 μg (Figure 1). These results confirmed that AMeX-processed tissue preserves DNA similarly to fresh tissue.

Total amounts of spooled DNA extracted from 50 sections, each 20 μm thick, of AMeX-processed tissues are listed in Figure 2. A linear correlation was observed between the area of tissue and the amount of DNA. In general, the total amount of spooled DNA from 50 sections, each 20 μm thick, was approximately 8 μg/mm^2 in malignant lymphoma cases, regardless of histologic subtype.

The electrophoretic patterns of restriction fragments of DNA on agarose gel stained with ethidium bromide were identical between AMeX-processed tissue and fresh materials from the same case. Goelz et al[2] showed that the electrophoretic patterns of DNA from formalin-fixed paraffin-embedded tissues digested with restriction enzymes were similar to those of unfixed fresh tissues when the tissues were fixed and embedded for routine histopathologic examined immediately after surgical removal and used for DNA analysis without long storage periods. However, the electrophoretic patterns of DNA from formalin-

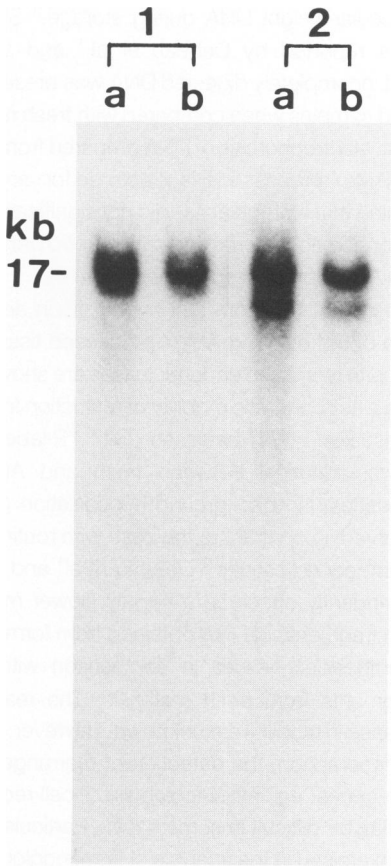

Figure 4. *Southern blotting of restriction fragments of DNA from AMeX-processed (a) and fresh (b) tissues of two cases of B-cell lymphomas (lanes 1 and 2). Ten micrograms of DNA was digested with BamHI endonuclease, electrophoresed on agarose gel, transferred to nitrocellulose, hybridized with a ^{32}P-labeled JH probe, and autoradiographed. The molecular weight of the germline band is indicated in kilobase pairs (kb).*

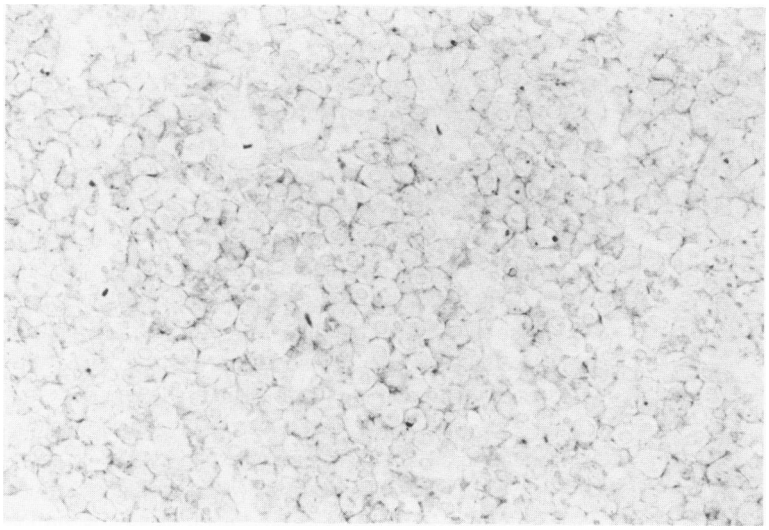

Figure 5A. *An AMeX-processed tissue section of adult T-cell lymphoma stained with CD 4 (anti-Leu3a) by the ABC method. Almost all the pleomorphic tumor cells show membrane staining (magnification, ×175).*

fixed and paraffin-embedded tissues stored for prolonged periods of time were different from those of DNA obtained from fresh tissue due to the degradation and low yield of high–molecular-weight DNA during storage.[2] Similar results were reported by Debeau et al,[1] and a certain amount of incompletely digested DNA was present in formalin-fixed samples when compared with fresh materials. In contrast, electrophoresis of DNA prepared from paraffin blocks of AMeX-processed tissues stored for varying periods revealed that storage at 4 C did not significantly affect the electrophoretic patterns, indicating good preservation of DNA in AMeX-processed tissue (Figure 3).

The results of Southern blot hybridization analysis of DNA from both fresh and AMeX-processed tissues after digestion with restriction endonucleases are shown in Figure 4. The electrophoretic mobility of restriction fragments of DNA visualized by hybridization with ^{32}P-labeled DNA probes was identical between fresh and AMeX-processed tissues. No background hybridization ("noise") was observed, in contrast to the case with routinely fixed paraffin-embedded tissues.[2] Debeau et al[1] and Goelz et al[2] independently observed a slightly slower mobility of restriction fragments of DNA obtained from formalin-fixed paraffin-embedded tissues in comparison with that of DNA fragments from fresh materials. The reasons for this decrease in mobility are unknown. However, because of this phenomenon, the detection of rearranged bands of certain genes, eg, immunoglobulin, T-cell receptor, or c-*myc*, may be difficult in some cases. Particular caution must be exercised in the analysis of immunoglobulin or T-cell receptor gene rearrangements using DNA extracted from formalin-fixed paraffin-embedded tissues because the changes in mobility of bands are of critical diagnostic importance. Because DNA from AMeX-processed tissue

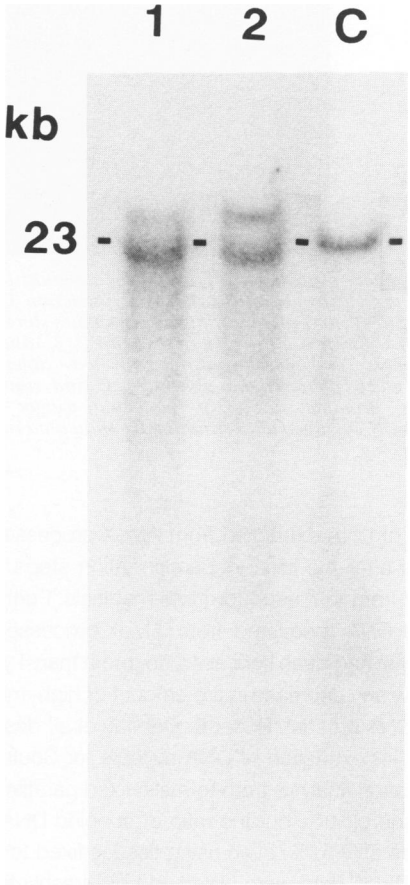

Figure 5B. *Southern blotting of DNA extracted from AMeX-processed tissue section (1) and fresh tissue (2) of the same case and HL-60 cultured cells as a control (c) after digestion with restriction endonucleases. Ten micrograms of DNA was digested with BamHI, electrophoresed, transferred to nitrocellulose, hybridized to a ^{32}P-labeled TcRβ probe, and autoradiographed. The molecular weight of the germline band is indicated in kilobase pairs (kb).*

showed electrophoretic mobility similar to that of DNA from fresh tissue, DNA from AMeX-processed tissue appears suitable for such analysis.

The results of immunophenotyping and genotyping of the 25 cases of lymphoma used in this study were in complete agreement. One example is illustrated in Figure 5. In this case, immunohistochemical staining clearly demonstrated the T-cell nature of this tumor, and gene analysis of DNA from the same block showed rearrangement of the TcRβ gene only.

In recent years, gene analysis has been increasingly used in the field of cancer research. Alterations of protooncogenes or other genes play an important role in the oncogenesis and progression of certain cancers.[7-9] For example, amplification of the N-*myc* gene in neuroblastoma has been shown to be associated with tumor aggressiveness, and thus with poor prognosis.[10,11] If only DNA from fresh tissue were suitable for such gene analysis, it would be difficult to accumulate a large number of cases in a short period of time. To overcome this problem, extraction of DNA was attempted from formalin-fixed paraffin-embedded tissues processed for routine histopathologic examination. After it had been shown that DNA extraction from formalin-fixed tissues was feasible, a few retrospective studies were performed using DNA extracted from formalin-fixed paraffin-embedded tissues and analyzing the correlation between gene amplification and the biologic behavior of malignant tumors.[3,4] However, these studies used dot- or slot-blot hybridization analysis instead of Southern blot hybridization because the extracted DNA revealed degradation.

Tsuda et al[4] predicted that analysis of DNA extracted from paraffin-embedded tissues could be extended to examinations of specific gene deletion and rearrangement by Southern blot analysis once an appropriate fixation and embedding method had been established for histological examination without extensive degradation of DNA. The AMeX method, with its simplicity and versatility as a multipurpose tissue-processing method, seems to be an answer to that expectation.

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