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Technical Advance

Fixation Conditions for DNA and RNA *in Situ* Hybridization

A Reassessment of Molecular Morphology Dogma

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Neutral buffered formalin (NBF) (4% neutral buffered formaldehyde) has been advocated by most investigators as the primary fixative of choice for *in situ* hybridization (ISH), and specific anecdotal cautions interdicting the use of precipitating fixatives, which otherwise may offer certain advantages such as superior nuclear detail, are common. Few systematic studies addressing ISH fixation conditions have been published. We reasoned that heavy metals present in some precipitating fixatives may compromise duplex formation during ISH. Cell lines containing known viral gene content (CaSki, 200 to 600 human papilloma virus 16 copies/cell, and SiHa, 1 to 2 human papilloma virus 16 copies/cell) and two negative cell lines (K562 and MOLT 4) were expanded to $>10^{10}$ and pellets fixed in NBF, zinc formalin, B5, and Bouin's and Hollande's solutions, and subjected to DNA ISH using biotinylated genomic probes. Ten tissue biopsies fixed in both Hollande's and NBF solutions were also evaluated for human papilloma virus content using DNA ISH. Additionally, 17 cases of Hodgkin's disease fixed in B5 and formalin were compared for Epstein-Barr encoded RNA detection using RNA ISH with fluorescein isothiocyanate-labeled oligonucleotides. Catalyzed reporter deposition combined with Streptavidin-Nanogold staining and silver acetate autometallography (Catalyzed reporter deposition-Ng-autometallography ISH) and a conventional indirect alkaline phosphatase method were used for detection for both DNA and RNA. Contaminating heavy metals entrapped in fixed tissues were removed by two exposures to Lugol's iodine. Results for both DNA and RNA ISH comparing B5 and NBF fixatives were virtually identical. Hollande's, Bouin's, B5, and zinc formalin fixed tissue showed results indistinguishable from NBF fixed tissue in DNA ISH. Precipitating fixatives such as B5 and Hollande's solution may be used for DNA and RNA ISH under appropriate conditions. *(Am J Pathol 1998, 152:35–41)*

The merging of nucleic acid detection with optical visualization through the technique of *in situ* hybridization (ISH) has spawned a new discipline, molecular morphology. Simultaneous visualization of a nucleic acid target in the context of morphology is a powerful tool for both understanding basic disease processes and ultimately for clinical diagnosis. Both DNA and RNA targets can be probed using this technology, and two decades of application attest to its use.

Fixation conditions for *in situ* hybridization are extremely important as integrity of the DNA or RNA target is fundamental to the technique's success. Although anecdotes addressing fixation can be found throughout the pertinent literature, careful systematic studies are difficult to identify. Nuovo and others[1–3] have performed three such systematic studies and concluded that neutral buffered formalin (NBF) is superior to other fixatives, especially Bouin's solution, for ISH. Other fixatives such as methanolic Carnoy's solution have been promulgated, but most authors advocate the use of NBF, as this fixative has functioned for most laboratories as the "substrate of

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pathology."[4] Although NBF is certainly the most commonly used fixative for tissue, alternative solutions are preferred by some investigators to either complement or replace formalin fixation. In particular, the use of precipitating fixatives, eg, B5 and Hollande's solutions, zinc formalin, or new proprietary fixatives such as Histochoice may offer certain advantages, such as improved nuclear detail or superior preservation of certain antigens for other techniques, eg, immunohistochemistry. The literature available on ISH, although principally anecdotal, generally speaks disparagingly of such alternative reagents.[4]

We reasoned that interference with either proteinase digestion or target/probe duplex formation may occur as a consequence of entrapped metals or other inhibitors in tissues processed with precipitating fixatives. The removal of such inhibitors before *in situ* hybridization should abrogate these deleterious effects. We tested this hypothesis with two systems using multiple cell lines and human tumor tissue biopsies containing human papilloma virus (HPV) for DNA: DNA ISH and RNA ISH to detect Epstein-Barr encoded RNA (EBER) expression in a series of Hodgkin's disease.

Materials and Methods

DNA:DNA ISH

Cell lines obtained from the American Type Tissue Culture Collection were used to assess the affect of fixation on DNA:DNA hybridization. CaSki is a cell line derived from human cervical carcinoma that contains approximately 200 to 600 copies of HPV 16 per nucleus with at least 11 chromosomal integration sites for the virus.[5,6] The SiHa cell line was derived from cervical carcinoma and contains 1 or 2 copies of HPV 16 per cell.[7] The erythroleukemia cell line K562 and the lymphoblastoid cell line MOLT4 were used as negative controls; K562 and MOLT4 were grown in suspension and CaSki and SiHa were harvested from monolayers as described below. Tissue biopsies fixed in both NBF and Hollande's solutions were also evaluated for both HPV subtypes, cytomegalovirus (CMV), adenovirus, Herpes virus, and Epstein-Barr virus DNA.

CaSki and SiHa cells were grown to confluence in large tissue culture flasks and removed from the culture surface with trypsin, yielding >1 × 1010 cells for study.

Paraffin blocks were prepared from cell cultures in two ways. First, approximately 4 × 10^6 culture cells were centrifuged, the pellets resuspended in thrombin-clotted AB serum, and the clots fixed for 4 hours in NBF, Hollande's solution, zinc formalin, B5, and Bouin's solutions. Similar preparations were made with 18 hours of fixation using only Hollande's and NBF solutions. The fixed cells in suspension clotted as above, then transferred to 70% alcohol, held until processing through graded alcohols and xylene, and paraffin embedded. Unfixed cell pellets were also suspended in Hollande's and NBF solutions for 15 hours of fixation, then subsequently pelleted via centrifugation, suspended in a thrombin/serum clot, and the clot transferred to 70% alcohol for processing and embedding in paraffin. Finally, to avoid the potential for target degradation by endonucleases in serum, cells fixed for 24 hours were also agar embedded. Fixed cells were washed with Hanks' balanced salt solution as above. Agarose (1%) in sterile water was boiled in a microwave oven and allowed to cool and maintained at 55°C in a water bath. Liquid agar was added to an equal volume of fixed cells, briefly vortexed, transferred to an ice bath for 10 minutes, and the pellet removed and processed for paraffin sections as above.

DNA:DNA ISH was performed using catalyzed reporter deposition[8–11] linked to Streptavidin-Nanogold[6,12–23] followed by silver acetate autometallography (CARD-Ng-AMG).[24,25] Sections were deparaffinized in two changes of xylene for 10 minutes each. Sections (4 μm) of tissue fixed in NBF were then washed in two changes of absolute isopropyl alcohol for 5 minutes each after xylene deparaffinization and allowed to air-dry. Sections fixed in the alternative solutions were dehydrated after xylene deparaffinization through graded alcohols, exposed to 1% Lugol's iodine solution for 5 minutes, washed in double-distilled water, decolorized for approximately 3 seconds in 2.5% sodium thiosulfate, and followed by thorough washing in double-distilled water. Sections of tissues fixed in any solution other than NBF received two exposures to Lugol's iodine. We attempted to use only one Lugol's treatment at the beginning of the procedure, but autometallography performance was compromised unless a second exposure following posthybridization stringency washes was used. The Lugol's iodine used was prepared in house by dissolving 18 g of potassium iodide (Fisher Scientific, Fairlawn, NJ) in 400 ml of distilled water, adding 9 g of iodine crystals (Fisher Scientific), and then adding another 500 ml of distilled water. Sections were dehydrated in graded alcohols, finishing with two 5-minute changes in absolute isopropyl alcohol. Alcohol dehydrated cytospins, gravity sedimentation preparations, and rehydrated paraffin sections were digested with proteinase K (0.01% in phosphate-buffered saline (PBS)) for 4 minutes at 37°C on a thermocycler plate and transferred to PBS for two washes, 1 and 5 minutes, respectively. Endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol for 30 minutes at ambient temperature. Sections were washed with double-distilled water and dehydrated through graded alcohols, finishing with two changes of absolute isopropyl alcohol, 5 minutes each. Prehybridization block was performed with 50% deionized formamide and 10% dextran sulfate in 2× SSC for 5 minutes at 50°C. Excess blocking solution was tapped off the slide and 20 μl of biotinylated genomic probe for HPV 16/18 (Enzo Diagnostics, Farmingdale, NY) applied to the section and coverslipped. Biotinylated HPV 6/11 genomic probe (Enzo Diagnostics) was used as negative control for the cell lines (Enzo Diagnostics). The probe and target were denatured together on a thermocycler for 4 minutes at 95°C and incubated at 37°C in a humidified chamber for 2 hours. Stringency washes consisting of 2× SSC (prepared from 20× SSC stock; Sigma, St. Louis, MO) for 5 minutes at ambient temperature, followed by two high stringency

washes, 0.2× SSC at 37° and 2× SSC for 5 minutes at ambient temperature. Initial coverslip removal proceeded stringency washes using gentle passage of the slides through 2× SSC solution until the coverslip was observed to slide off of the slide.

Detection of biotinylated hybridized probe was achieved through the use of the catalyzed reported deposition linked to Streptavidin-Nanogold followed by silver acetate autometallography procedure (CARD-Ng-AMG).[24,25] Slides prepared as above through stringency wash steps were transferred from 2× SSC to 1% Lugol's iodine solution for 5 minutes, washed thoroughly with double-distilled water, and decolorized for approximately 3 seconds in sodium thiosulfate. Slides were then thoroughly washed in double-distilled water, followed by 5 minutes in PBS, and preblocked with 4× SSC containing 0.05% Tween 20 (Boehringer Mannheim, Indianapolis, IN) for 5 minutes at 37°. Streptavidin-biotinylated peroxidase preformed complex (Dako, Carpinteria, CA) 1:300 was applied to the sections and allowed to incubate at 37° for 30 minutes, followed by two washes of 3 minutes each in PBS at ambient temperature. After a 5-minute wash in 0.5 mol/L Tris-HCl, pH 7.4, lyophilized biotinylated tyramide (NEN Life Science Products, Boston, MA) solubilized with 125 μl ethanol, additionally dissolved with the manufacturer's amplification buffer and an equal volume of ultrapure water, applied to the section, and allowed to incubate for 10 minutes at ambient temperature. The slides were then washed in 100 mmol/L Tris-buffered saline (TBS), pH 7.4, twice for 5 minutes each at ambient temperature. Sections were then incubated with Streptavidin-Nanogold® (Nanoprobes, Stony Brook, NY), at a concentration of 1:3000 in 1% bovine serum albumin (BSA) in TBS for 1 hour at ambient temperature. The sections were then washed in TBS for five minutes at room temperature, followed by two washes of 5 minutes each in TBS containing 0.1% fish gelatin (Aurion, Wageningen, The Netherlands) in TBS, and followed by fixation for 2 minutes in 2% freshly filtered glutaraldehyde (Merck, Darmstadt, Germany) in TBS. Following four washes of 5 minutes each in Nanopure water, silver acetate autometallography was performed as previously described.[15] Silver acetate solution contained 100 mg of silver acetate in 50 ml of ultrapure water (magnetic stirring at room temperature required to fully solubilize). A separately prepared solution of hydroquinone containing 200 mg in 50 ml of citrate buffer, pH 3.8 (trisodium citrate dihydrate 23.5 g and citric acid monohydrate 23.5 g in 850 ml of Nanopure water; pH adjusted to 3.8 with citric acid). The two solutions were prepared during the Streptavidin-Nanogold incubation, not combined, and stabilized at 22°C. When the washed sections were ready for autometallography, the silver acetate and hydroquinone solutions were mixed, and the slides immediately immersed in the autometallography solution for 8 minutes at 22°C. The silver development was stopped in distilled water. Sections were then subsequently counterstained with nuclear fast red (Enzo Diagnostics), dehydrated in graded alcohols and xylene, and coverslipped.

RNA ISH

The system used for comparative fixative evaluation of RNA ISH was a series of 17 tissue biopsy specimens of Hodgkin's disease, some of which were known to contain EBER as evaluable target RNA. Hodgkin's cases with tissues fixed in both formalin and B5 were comparatively studied. Section rehydration, Lugol's pretreatment of B5 fixed tissue, endogenous peroxidase blocking, and proteinase K digestion were as per DNA ISH described above. Prehybridization block was done at 37°C; no denaturation was performed. Fluorescein isothiocyanate (FITC)-haptenated oligonucleotides were applied to the sections, coverslipped, and hybridized at 37°C for 2 hours. Posthybridization stringency washes were as per DNA ISH.

Two FITC-labeled oligonucleotide probes specific for the EBER-2 of Epstein-Barr virus were used for RNA ISH (sequences provided by Ventana Bioteck, Tucson, AZ). Sections were washed twice, 3 minutes each, in PBS and then incubated for 30 minutes in monoclonal mouse anti-FITC (Boehringer Mannheim), 1:1000 in PBS, followed by two washes in PBS, 3 minutes each. Biotinylated goat anti-mouse IgG (1:1000 in PBS) was incubated for 20 minutes at room temperature with the sections followed by two 3-minute washes in PBS, and streptavidin-biotinylated peroxidase and subsequent detection reagents as per DNA ISH above.

Colorimetric ISH

Conventional two-step colorimetric *in situ* hybridization was performed using an automated ISH instrument (GEN II, Ventana Bioteck) and alkaline phosphatase-based detection. All steps of the procedure following rehydration, Lugol's treatment of B5 and Hollande's fixed tissues including protein digestion, denaturation, reagent applications, and washes, were controlled by the instrument. Sections were digested using protease I (Ventana Bioteck) for 8 minutes at 37°C. Whole cell gravity sedimentation preps digested with protease II (Ventana Bioteck). Detection of hybridized biotin or FITC-labeled probes was done using a nitro blue tetrazolium-alkaline phosphatase detection kit supplied by the manufacturer. Mild denaturation of RNA target by heating to 75°C was used to eliminate any secondary structure formation. Section rehydration, Lugol's pretreatment of B5-fixed tissue, protease digestion were as per DNA ISH described above. For RNA ISH, the sequence began with FITC-labeled oligonucleotides. FITC-haptenated oligonucleotides were applied to the sections, coverslipped, and hybridized at 37°C for 1 hour. Posthybridization stringency washes using 2× SSC for 4 minutes (twice) and 1× SSC for 4 minutes at 42°C were performed. Mouse monoclonal anti-FITC 1:1000 (Boehringer Mannheim) in Ventana reagent buffer for 20 minutes, was followed by Ventana detection (biotinylated anti-mouse IgG/streptavidin alkaline phosphatase/nitro blue tetrazolium). Oligo-dT probe was used as positive RNA control; nonsense oligos were used as negative controls (NovoCastra/Vector; Burlingame, CA).

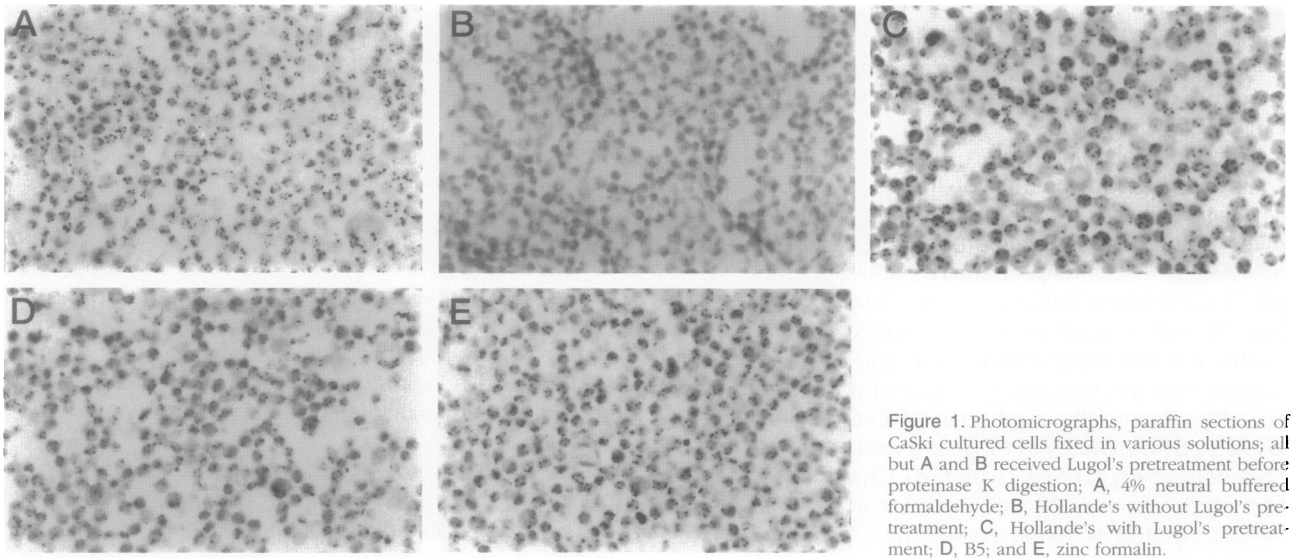

Figure 1. Photomicrographs, paraffin sections of CaSki cultured cells fixed in various solutions; all but A and B received Lugol's pretreatment before proteinase K digestion; A, 4% neutral buffered formaldehyde; B, Hollande's without Lugol's pretreatment; C, Hollande's with Lugol's pretreatment; D, B5; and E, zinc formalin.

Results

Results for DNA and RNA ISH are summarized in Tables 1 and 2, and illustrated in Figures 1 through 3.

DNA ISH demonstrated intense, large granular staining of each nucleus of cells derived from CaSki cell line using CARD-Ng-AMG. In paraffin sections, as sections of nuclei were present containing varying amounts of target, the granular precipitates were confined to the nucleus and were less frequent but prominent (Figure 1A). These results were not simply silver-stained nucleolar-organizing regions or other artifacts, as no staining was observed in preparations using biotinylated HPV 6/11 or HPV 31/33/35 genomic probes.

Figure 1 also illustrates the importance of Lugol's iodine pretreatment for non-NBF-fixed tissue. In the absence of this reagent, which presumably extracts residual metals and other materials deleterious to the hybridization or proteinase digestion reaction, virtually no hybridized signals were observed (Figure 1B). If pretreatment was used, the number of positive events and their intensity was indistinguishable from parallel samples prepared in NBF (Figures 1, C to E). As summarized in Table 1, by using such pretreatment, a variety of fixatives yielded preparations that were performed equally to NBF, including Hollande's solution (Figure 1C), B5 solution (Figure 1D), and zinc formalin (Figure 1E).

Table 1. *In Situ* Hybridization (HPV 16/18) CARD-Ng Method

Fixative	MOLT4	K562	CaSki	SiHa
B5	−	−	++++	+
Zinc formalin	−	−	++	+
Formalin	−	−	+++	+
Hollande's	−	−	++++	+
Bouin's	−	−	++	+

−, negative; +, 0 to 25% of cells positive; ++, 25 to 50% of cells positive; +++, 50 to 75% of cells positive; ++++, 75 to 100% of cells positive.

This effect was also observed for tissue biopsy samples (Figure 2). The specimen illustrated was positive for HPV 16/18 by extraction dot blot assay, which was corroborated for positive ISH in NBF-fixed tissue sections. Corresponding Hollande's-fixed biopsy material from this case showed only spotty, focal reactivity without Lugol's iodine pretreatment (Figure 2A), but this effect was entirely abrogated by pretreatment with Lugol's solution

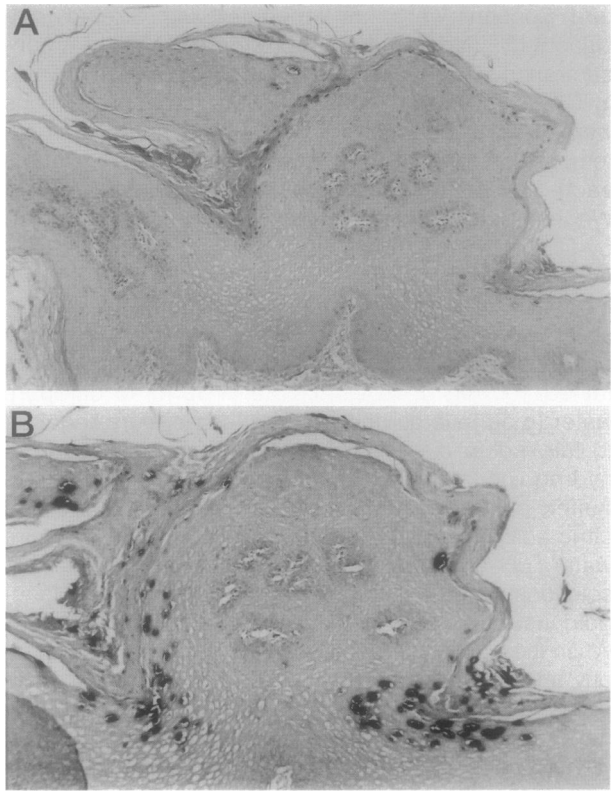

Figure 2. Photomicrographs, condyloma fixed in Hollande's solution (**A** and **B**). Lugol's iodine pretreatment followed by sodium thiosulfate was used only for **B**.

Table 2. EBER *in Situ* Hybridization in Hodgkin's Disease

	CARD-Ng-AMG				Streptavidin-alkaline phosphatase			
Case	Formalin small cells	Formalin large cells	B5 small cells	B5 large cells	Formalin small cells	Formalin large cells	B5 small cells	B5 large cells
1	++++	++++	++++	++++	T	+++	++	++++
2	–	–	–	–	–	–	++	–
3	–	–	–	–	T	–	T	–
4	–	–	T	–	–	–	–	–
5	–	–	T	–	++	–	++	–
6	+++	–	–	–	–	–	–	–
7	–	–	T	–	–	–	–	–
8	T	–	T	–	T	–	T	T
9	T	–	T	–	–	–	–	–
10	T	–	T	–	+	–		
11	+	–	++	T	–	–	–	–
12	T	–	–	–	–	–		
13	–	–	T	–	–	–	T	–
14	++++	++++	++++	++++	++	+++	++	++++
15	–	–	T	–	T	–	–	–
16	T	–	–	–	–	–	–	–
17	++	++	+	++++	+	++	+	++

Abbreviations: T, trace, only a few nuclei positive.

followed by sodium thiosulfate (Figure 2B). The negative controls (K562 and MOLT 4 cell lines) were negative (Table 1). There was no significant background staining (Figure 1).

DNA:DNA ISH results observed using the conventional indirect alkaline phosphatase method generally reflected the lesser sensitivity of this approach. SiHa cells were negative, and CaSki cells showed only a few positive cells in each field.

Table 2 summarizes the results for RNA hybridization. In this series of 17 Hodgkin's disease cases, the overall agreement between NBF- and B5-fixed tissues for both the automated method and the CARD-Ng-AMG technique was excellent (Table 2). We noted qualitatively a greater intensity of staining in B5-fixed material as compared with sections of formalin fixed tissue in the alkaline phosphatase based colorimetric method (Figures 3A and B). Using the CARD-Ng-AMG method, these differences were not identified, probably because of greater sensitivity of the latter technique or perhaps because of an unidentified but systematic bias in formalin processing used for lymphomas at the institution preparing the study set (Cleveland Clinic).

Two types of staining patterns were observed. The most prominent staining consisted of intense nuclear expression in Reed-Sternberg cells and large mononuclear cell variants. When large cells only were considered, both methods gave very similar results, probably due to the extremely high EBER copy known to be present in such cells. The second EBER pattern was a distribution restricted to scattered small lymphocytes but no labeling of large nucleolated cells.

Discussion

NBF, as a fixative solution, has been called the substrate of pathology.[4] Indeed, this fixative has served pathology very well for decades. Other fixative solutions may be preferred by some pathologists or pathology groups for a variety of reasons, including safety concerns regarding harmful effects of formalin or to specific advantages accorded the use of alternative fixative solutions. Precipitating fixatives such as Hollande's, Zinc formalin, and B5 solutions often produce superior nuclear detail or enhanced preservation of antigenicity, and at least for some antigens, no or minimal proteinase digestion for immunohistochemical detection of antigens requiring enzyme digestive pretreatment for selected non-NBF-fixed tissues may also account for such preferences.

The few reports that have systematically examined fixation conditions for *in situ* hybridization have assumed that postfixation processing and preparation of sections should be identical for NBF and alternative fixation.[1–3] Much is known about the variables affecting duplex formation and stability in ISH. Probe complexity, symmetry, base content, buffer stringency, temperature, and formamide concentration all interact and contribute to successful ISH.[4,26–29] But little appears to be known regarding the effect of heavy metals on this process. Following an empirical observation in which a B5-fixed tissue was inadvertently processed along with NBF sections in an EBER RNA ISH experiment in which strong signals were anecdotally observed, we designed a study to systematically evaluate DNA and RNA *in situ* hybridization fixation conditions. Because our B5-fixed tissues are usually subjected to Lugol's treatment to remove brown-black precipitates, it was logical to consider the extraction of heavy metals via iodine pretreatment as the ameliorating factor. The study reported herein used both expanded cell culture lines fixed in a variety of fixatives, as well as human Hodgkin's disease tissue specimens containing EBER.

The CARD-Ng-AMG method could perhaps account for the differences between our study and previous reports. This technique is extraordinarily sensitive, detecting as little as one HPV gene copy/cell.[25] The Hodgkin's disease cases evaluated for EBER content using an al-

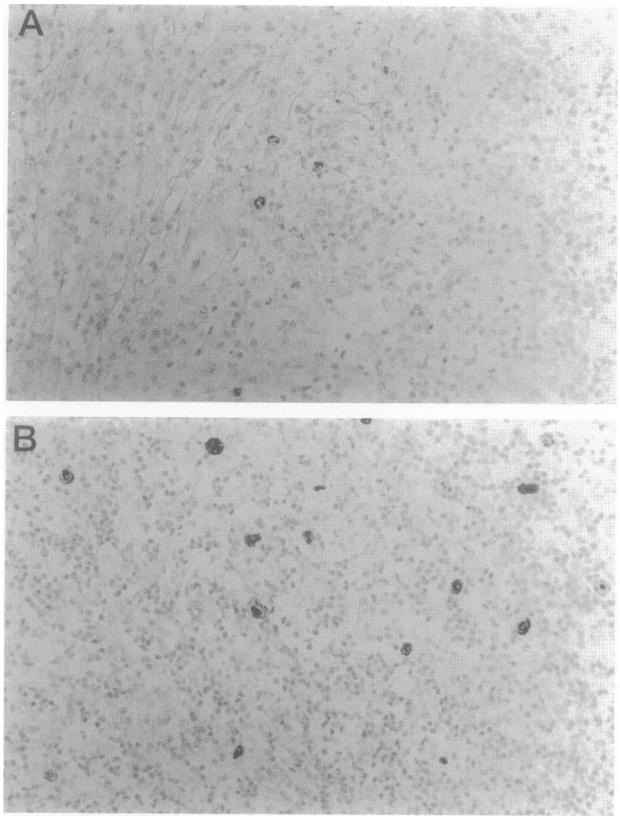

Figure 3. Photomicrographs, adjacent paraffin sections of Hodgkin's disease fixed in formalin (**A**), and B5 (**B**) solutions. **B** was pretreated with Lugol's iodine solution. Both were subjected to RNA ISH using a colorimetric alkaline phosphatase-based method and nitro blue tetrazolium detection.

kaline phosphatase based system of apparently lesser sensitivity demonstrated that staining intensity of cells fixed in B5 appeared qualitatively stronger than cells from the same biopsy tissue fixed in neutral buffered formalin (Figure 3). These differences in intensity were not apparent using the CARD-Ng-AMG technique.

The precise mechanism whereby iodine pretreatment ameliorates ISH results in non-NBF-fixed tissues is uncertain. The degradation of DNA, as assessed by digestion patterns with restriction endonucleases, is much better preserved in extracted DNA from tissues fixed in buffered formalin.[2,3,30,31] However, protection of DNA integrity alone does not explain these differences, as non-buffered formalin also produced degraded DNA but ISH results similar to NBF fixed tissue.[3] Presumably, iodine, or perhaps an interaction of iodine and sodium thiosulfite, removed an identified factor that compromises duplex formation during *in situ* hybridization. Heavy metals, such as mercury in B5, zinc in zinc formalin, and copper in Hollande's solutions, may partially explain this empirical observation but obviously do not account for our data with Bouin's-fixed tissues. We attempted to use only one Lugol's treatment at the beginning of the procedure, but autometallography performance was compromised unless a second exposure following posthybridization stringency washes was used. This was empirical, and we don't have a clear answer to explain it.

Single DNA target copy was detectable using the CARD-Ng-AMG system; the multiple steps involved in this procedure are quite laborious, and automation of this method should be pursued. Furthermore, the system used for evaluating fixation conditions for RNA ISH contains relatively large abundance of EBER target, and its applicability to lower level mRNA species detection should be further evaluated.

Acknowledgments

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