Identification of surgical biopsy borders by use of India ink

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Summary: Separation of surgical biopsy borders from artifactual borders created during trimming of biopsy specimens is necessary to avoid misinterpretation of histologic borders. Misinterpretation of a contaminated trimming border as a surgical border may lead to additional surgery and excessive removal of normal tissue. Likewise, a neoplasm may regrow locally or metastasize if a surgical border infiltrated with neoplastic cells is falsely assumed to be an artifactual trimming border. The use of India ink for distinguishing between surgical biopsy borders and artifactual borders was evaluated. Ten normal tissue specimens from 8 types of tissue (skin, small intestine, urinary bladder, bone, muscle, lung, large intestine, and uterus) were obtained from freshly euthanatized dogs. The specimens were painted with India ink and examined for adherence of the ink to the cut surface of the specimen. Adherence of the ink was observed in all specimens with the exception of the cut surface of the lung. Twenty-five biopsy specimens from dogs with clinical cases of disease were similarly painted with India ink and evaluated. Twenty-two were identified as neoplastic and 3 as inflammatory lesions. Wedges of tissue were obtained from the center of the biopsy specimens to purposely create borders that contained neoplastic tissue. These positive controls were painted with India ink to evaluate the effect of the ink on the histologic appearance of the neoplastic cells. Distortion or alteration of the cellular architecture was not observed in any of the normal specimens, specimens from dogs with clinical cases, or positive controls. The use of India ink for delineation of biopsy borders is a simple technique that presents few technical difficulties. India ink should be applied routinely to the excised borders of all excisional biopsy specimens to facilitate identification of the surgical border.

Although numerous diagnostic and therapeutic options have become available to the oncologist in the last 2 decades, surgery continues to maintain an important role in the diagnosis and treatment of many neoplasms. Total excision of neoplastic masses is mandatory for cure, and as excision nears completeness, the benefits of adjuvant radiation therapy and chemotherapy are enhanced. Total excision requires that no neoplastic cells remain in the animal. The attempt to remove all of the neoplastic tissue usually results in the removal of a generous amount of surrounding normal tissue with the neoplasm. The extent of normal tissue excision may be offset by the need to preserve function and appearance. If the surgeon conservatively excises tissue in an effort to preserve function or minimize disfiguration, neoplastic tissue may remain in the body, especially if the neoplasm is locally highly invasive such as cutaneous mast cell tumors. Accurate and consistent delineation of surgical borders is, therefore, of paramount importance in oncologic surgery.

Typically, processing of biopsy tissue includes trimming the tissue to fit into a plastic cassette approximately 5 mm × 25 mm × 30 mm. Sharp borders created by the trimming knife during specimen processing, in addition to occasionally distorting the orientation of the section, may create confusion as to whether the border viewed microscopically is a surgically created border or a result of specimen processing. Another artifactual processing border can be created if the tissue tears, fragments, or retracts during sectioning of the paraffin block that contains the embedded biopsy tissue. If the histopathologist observes neoplastic cells extending to the borders of the section, a decision must be made as to whether this border is a surgical border or one created by trimming or sectioning during specimen processing. A surgical border containing neoplastic cells is referred to as a contaminated or "dirty" margin and dictates additional therapy for the animal; however, a contaminated margin created by trimming may be inconsequential if the surgical margin is "clean." The wrong decision has potentially serious consequences. The animal may undergo unnecessary
Figure 1—Photomicrographs of normal cortical bone (A), small intestine (B), skin (C), and lung (D) after India ink has been applied. Observe the lining of the pleura by the ink, whereas no ink lines the cut surface of the lung. H&E stain; bar = 50 μm.

Further surgery, chemotherapy, radiation, or other procedure if a contaminated trimming margin is assumed to be a surgical border, or the neoplasm may regrow locally or metastasize if a contaminated surgical border is falsely assumed to be a postsurgical trimming border. Postoperative evaluation of the completeness of excision is not easily accomplished. Marking the borders of biopsy specimens with suture tags is a simple but inaccurate technique. Suture tags may help the pathologist identify which surface of the biopsy specimen is the excised surface, but the extent of the excised surface may not be obvious. Furthermore, suture tags must be removed prior to processing of the specimen; removal of the suture may result in loss of orientation.

Accurate techniques for assessing surgical borders usually involve specialized sectioning and labeling of the excised mass. These techniques are rarely used by veterinarians because of the inconvenience imposed by precise specimen preparation, special training of surgical personnel and histology technicians, and mandatory personal communication between the surgeon excising the tumor and the pathologist examining the biopsy specimen. A simple, reliable, universal method for distinguishing between surgical and processing borders is needed.

Application of India ink to the excised surgical margin prior to formalin fixation has been suggested as a simple, accurate, and consistent technique for separating surgical from processing borders. Dots of India ink have been used in histology laboratories to mark the inner surface of histologic sections for orientation purposes. India ink has also been used for marking specimens following limited resections of breast neoplasms in women and for orientation purposes in a histologically guided cutaneous tumor removal technique in lieu of mercurochrome and laundry bluing. India ink has been used in a similar fashion to the technique reported here in the examination of borders of excised canine bone tumors.

The purpose of the study reported here was to determine the ability of India ink to adhere to the cut surfaces of normal and neoplastic tissues and to
Figure 2—Photomicrographs of section of a mast cell tumor. Notice extension of the tumor to the inked border (A). H&E stain; bar = 50 μm. Higher magnification (B) of the same section. Notice no distortion or alteration of the cellular architecture by the ink; bar = 25 μm.

provide easy microscopic identification of incised borders.

**Material and Methods**

_Normal tissues_—Grossly normal tissue specimens of cortical bone (n = 10), uterus (n = 10), small intestine (n = 10), urinary bladder (n = 10), large intestine (n = 10), lung (n = 10), skin (n = 10), and skeletal muscle (n = 10) were obtained from freshly euthanatized dogs that had been used in another research project. The cut surface of each specimen was blotted free of blood, painted with India ink by use of a cotton swab, allowed to dry for 3 minutes, and fixed in neutral buffered 10% formalin at a formalin:tissue ratio of 10:1. Bone specimens were decalcified with hydrochloric acid. All specimens were sectioned in 6-millimeter thicknesses to allow proper fixation. Following fixation, the specimens were trimmed to include the inked surfaces, fixed in paraffin, and sectioned at a thickness of 6 μm. All specimens of the normal tissues were examined by the same pathologist (LWP). Specimens were evaluated for adherence of India ink to the tissue, microscopic alteration of the histologic pattern at the tissue-ink interface, distortion or enhancement of the cytologic characteristics, and artifacts produced by the ink.

_Abnormal tissues_—Twenty-five excisional biopsy specimens were obtained from 22 dogs admitted to the teaching hospital for excision of confirmed or suspected neoplasms. Clinical management of these dogs was in accordance with accepted oncologic principles and routine protocols used at the hospital. Following excision of the mass with a scalpel, the excisional border was painted with India ink and allowed to dry. Then, a small wedge was excised from the center of the mass in an attempt to create a purposely contaminated border (positive control). Only excised tumors that were large enough to allow this wedge of tissue to be removed without interfering with clinical interpretation and border evaluation were used for this purpose. The borders of the positive controls were painted with India ink and the excisional biopsy and positive control specimens were processed as described. Sharp excision was used in all cases instead of electroscalpel because of the potential for artifactual change with the use of electrosurgery. Painting and processing of all 25 abnormal tissues prior to formalin fixation was completed by 1 of 2 investigators (MCR, FAM) within 30 minutes following excision. Tissues were submitted for routine histologic examination with special request to cut into and microscopically examine the inked border. The clinical specimens were read by the pathologist on duty at the time of specimen submission. The purposely created neoplastic borders were evaluated by a third investigator (LWP) for presence of the India ink along the border, presence of tumor cells along the inked border, and any distortion of the cells or artifacts. The clinical and histologic diagnoses of the biopsy specimens were unknown to the pathologist (LWP) at the time of examination of the positive controls. Upon completion of examination of the positive controls, the histologic diagnoses of the positive controls were compared with the results of the previously diagnosed clinical specimens.

**Results**

_Normal tissues_—In all of the normal specimens, ink was lining the surgical border of the specimen with the exception of the cross section of lung parenchyma (Fig 1). Three specimens of lung had partial adherence of the ink to the cut surface while the remaining 7 retained no ink on the cut surface. Distortion or artifactual change was not
observed in any of the inked borders. Decalcification did not alter the inked border of bone specimens.

Abnormal tissues—Twenty-two of the clinical biopsy specimens were confirmed to be neoplasms (Appendix); 3 were identified as inflammatory lesions. Abnormal neoplastic or inflammatory cells were found on all positive controls at the inked border. None of the cells at the border were distorted (Fig 2 and 3). Lipomas were especially well delineated by the ink. India ink was observed in blood vessels within neoplastic tissues in 2 specimens; 1 was a mixed mammary tumor, the other a mast cell tumor. Histologically, tumor did not extend to the inked border in 5/14 clinical biopsy specimens because it was believed that the specimens were inflammatory masses or in 2/8 clinical biopsy specimens diagnosed histologically as benign masses. Extension of the neoplastic cells to the inked border was observed in 3/14 malignant masses. Comment on the presence or absence of the India ink was not made by the pathologist in 3/3 inflammatory masses, 6/14 malignant masses, and 4/8 benign masses. Only one sample section from each specimen was read.

Only 2 clinical biopsy diagnoses varied from the positive control diagnoses. In both instances, the positive control was identified as inflammatory tissue. Histologic examination of the entire tumor revealed neoplasia surrounded by inflammatory tissue. One tumor was an undifferentiated sarcoma and the other was a squamous cell carcinoma.

Discussion

Eight types of normal tissue were chosen to evaluate the ability of India ink to adhere to a wide array of tissues. The variety of neoplastic and inflammatory tissues was dictated by the clinical caseload during the course of the study.

Earlier, reports of the use of India ink advised use of Bouin's solution following painting with the ink.\(^5\) Bouin's solution is a tissue fixative prepared by combining formaldehyde, glacial acetic acid, and picric acid. Because of the lack of availability of Bouin's solution to most practitioners, we evaluated the use of India ink with neutral buffered 10% formalin. Bouin's solution is also explosive if allowed to dehydrate, posing a potentially serious hazard.\(^4\) Adherence of the ink to the tissue surface throughout the fixation and processing of the tissue demonstrates that Bouin's solution is unnecessary to the success of this technique.

The presence of ink within blood vessels of 2 neoplastic specimens was believed to have occurred because those specimens were painted before coagulation was complete. Ink was then drawn into the vessels by capillary action prior to coagulation of the intravascular blood. Blood within the vessels of tissue specimens should be allowed to clot prior to painting the tissue surface with India ink.

The 2 differences between the clinical diagnoses and the positive control diagnoses are believed to be attributable to sampling error. For each, the positive control diagnosis was inflammatory tissue. In the histologic examination report of the clinical biopsy specimens in both cases, peripheral inflammation was found around the tumors. Failure to incise deeply enough into the mass resulted in failure to include neoplastic cells in the positive control specimen.

The pathologist may not have commented concerning the presence or absenace of ink on some clinical biopsy specimens because it was believed that the specimens were inflammatory masses or benign masses that appeared to be completely excised. Additionally, the pathologist may have neglected to comment on the inked border, because of a failure to understand the meaning of the ink.

Failure of the ink to mark the cut surface of the lung parenchyma was attributed to relative lack of parenchyma to which the ink could adhere. The creation of tearing or fragmentation artifacts as described earlier is another possible cause for the lack of ink along the lung parenchymal border.

Lack of distortion of the margins of the specimen tissue by the ink is important because accurate identification of the cells along the border of the biopsy specimen is occasionally necessary for identification of the tumor type. Accurate identification of border cells is also important when inflammatory cells infiltrate the neoplastic mass because incision through the area of inflammation requires a different course of action than incision through neoplastic tissue. Additionally, many presumed neoplastic lesions may prove to be inflammatory on histologic examination. Because India ink does not distort inflammatory cells, diagnosis
of inflammatory lesions should not be impaired when the margin is inked.

One of the best referenced techniques for border identification is Mohs' surgery.\textsuperscript{1,10-13} This technique was developed in the 1930s by Dr. Frederic Mohs as a method of accurate mapping of cutaneous neoplasms. Originally, Mohs' surgery involved the application of zinc chloride paste to the wound surface of an excised tumor and mapping of the tumor and its bed. The excised tumor was then horizontally sectioned and the base examined for the presence of neoplastic cells. Any residual tumors was then plotted on the map and excised. The application of zinc oxide allowed excision of remaining neoplastic tissue without hemorrhage or pain. Mohs' surgery allowed for accurate identification of residual neoplastic tissue while sparing normal tissue from unnecessary excision; however, the technique is difficult, time-consuming, and useful only for skin tumors. In the 1970s, a fresh tissue modification was devised whereby the tumor was removed with local anesthesia and the specimen was processed in frozen sections.\textsuperscript{10,12} The use of zinc oxide was eliminated. The extensive mapping of the lesion and serial horizontal sectioning of the tumor were retained.\textsuperscript{10}

Compared with Mohs' surgery, India ink can help establish the presence of neoplastic tissue extending to the surgical border, but does not identify which specific border is involved. The inability to identify the exact portion of the border infiltrated with tumor may require a more extensive resection of the tumor bed than necessary. A multicolor system\textsuperscript{b} has been recently developed that allows the pathologist to identify as many as 5 different borders. Five tissue dyes (blue, black, red, green, and yellow) are applied to the base, both short-axis margins, and both long-axis margins. The 5 inks can be applied before or after fixation. Identification of neoplastic tissue extending to a border is then identified according to the color of the border.\textsuperscript{13} Reexcision can then be accomplished more accurately with minimal tissue loss.

The multicolor marking system presents a few inconveniences not encountered with the use of India ink. Application of the inks before fixation would require the veterinarian to be responsible for acquiring the specialized materials necessary to pain the biopsy specimen. Precise recording of the orientation details would also be required of the veterinarian. The system is also more expensive than use of India ink alone. Finally, although the excised border of a skin biopsy specimen can be subdivided into multiple areas to allow localization of remaining neoplastic tissue, a single color is sufficient to identify the microscopic border as a surgical border and whether or not tumor cells extend to that border. Future evaluation of the multicolor system in veterinary medicine is warranted.

On the basis of our findings, India ink is easily applied and used for delineating the surgical margins of biopsy specimens. India ink adheres well to all tissues except the cut surface of lung parenchyma. The biopsy specimen must be dry prior to inking the surface of the lesion, and the inked specimen should be dry prior to formalin fixation. Processing of biopsy specimens painted with India ink requires no special steps and gives an accurate assessment of the border of the specimen viewed microscopically. Trimming of the specimen to include the inked border is the only necessary technical point required of the histopathology technician. Presence of the inked border allows the pathologist to confidently indicate whether the border examined is a surgical border or a border created by trimming; however, total excision of the tumor cannot be guaranteed unless the entire specimen is examined.\textsuperscript{6} India ink apparently does not distort normal, inflammatory, or neoplastic cellular morphology. Biopsy specimens, superficial or deep, should be routinely painted with India ink prior to submission for histologic examination if knowledge of the completeness of excision is necessary for treatment planning.

Appendix

\textbf{Diagnosis of tissues biopsied}

- Mast cell tumor (n = 3)
- Sebaceous gland adenoma (n = 1)
- Fibrosarcoma (n = 2)
- Acanthomatous epulis (n = 1)
- Osteosarcoma (n = 1)
- Complex tubular adenoma (n = 1)
- Carcinoma (n = 1)
- Mixed mammary tumor (n = 1)
- Basal cell tumor (n = 1)
- Trichoepithelioma (n = 1)
- Perianal gland adenoma (n = 1)
- Lipoma (n = 3)
- Apocrine gland adenocarcinoma (n = 1)
- Squamous cell carcinoma (n = 1)
- Hemangiopericytoma (n = 1)
- Undifferentiated sarcoma (n = 1)
- Plasmacytoma (n = 1)

References


\textsuperscript{a}Davidson Marking System, Bradley Products, Bloomington, Minn.
Influence of wound shape on wound contraction in horses

Closure of a skin defect occurs as a result of 2 independent processes, contraction and epithelialization. Controversy has existed for years over whether the shape of a skin wound influences the rate of wound healing. Hippocrates taught that circular wounds healed very slowly. This observation led early surgeons to convert circular defects into defects with straight sides (eg, triangles, rectangles) in an effort to speed healing. This principle has persisted in modern surgery. Carefully controlled experimental studies of the effect of wound shape on wound healing have yielded conflicting results.

In our study, 3 sets of paired circular and square full-thickness skin wounds were made on the dorsum of the metacarpus (n = 48) of 8 horses. Each wound was 6.25 cm² in area. The wounds were treated topically with an ointment and nonadherent dressing, then bandaged with a snug elastic wrap. Wounds were photographed every other day until healing was complete. Wound areas were measured, and exponential and linear wound healing models were applied to the wound healing data generated. Wound healing variables measured for each wound were: number of days to healing, maximal size attained, rate of wound contraction (calculated by use of first-order and linear models), final wound size, and percentage of wound that healed by contraction.

The exponential model fit the data significantly better than the linear model. The maximal size attained by circular wounds was significantly smaller than the maximal size attained by square wounds. Wound shape did not influence the rate of wound healing. On the basis of our findings, we concluded that conversion of circular defects to square defects would not speed wound healing.—J. B. Madison and R. R. Gronwall in Am J Vet Res 53 (September 1992).