Histograph September 2003 Issue

Editorial

Welcome to the September issue of Histograph. In side you will find Review articles on "Sentinel Nodes in Breast Cancer" and "Iron Histochemistry". The Histonet (http://lists.utsouthwestern.edu/mailman/listinfo/histonet) is an invaluable source of experience and information and the article on Microbiology Controls has been purloined from this listserver.

This issue's Safety section deals with mountant media hazards and in particular Dibutyl phthalate. There is also a thought provoking news item on Picric Acid from the Daily Telegraph. It is probably timely that we all check our laboratory stocks of Picric Acid for drying.

The "Stain to Try" is the Harada's modification of the ZN stain which is reported to be more sensitive for the identification of mycobacteria.

There are also several items in the "Abstracts from the Literature that should be of interest.

The final issue for 2003 will include the "weird and wonderful" of Histopathology such as chicken antibodies and supermarket supplies for the mortuary.

Finally, the plans for next year's inaugural National Histotechnology Meeting in Sydney are well underway. Our president will explain more in his message.

What a Hoot!!!

I am excited!!

Tony Henwood
Editor
Histopathology
The Children's Hospital at Westmead
Anthonyh@chw.edu.au

Sentinel Nodes in Breast Cancer - A Review

Sentinel lymph node biopsy is an important treatment option for breast cancer patients, as it can accurately predict axillary status (1). Sentinel nodes have a higher chance of containing metastases than do non-sentinel nodes (2). A study using dye with or without radioisotope showed the accuracy and sensitivity of sentinel node biopsy to be 97% and 94%, respectively. Based on these results, in one institution axillary lymph node dissection was eliminated in patients with intraoperatively negative sentinel node biopsy (1). Of a total of 358 sentinel node negative cases, no patient developed an axillary relapse after 21 months follow-up. The results indicated that an intraoperative negative sentinel node biopsy without further axillary lymph node dissection might be a safe procedure when strict sentinel node biopsy is performed (1).

Early outcome data in sentinel lymph node biopsy suggest no adverse outcome for patients with metastases no larger than 2.0 mm, a finding aligned with the current definition of micrometastasis. When sentinel lymph nodes are sliced at 2.0-mm intervals and totally embedded, the probability of identifying all metastases >2.0 mm is high. Using reasonable sampling strategies, minute metastases have a nearly equal chance of being missed or detected. New staging guidelines have established a lower limit for micrometastases and defined metastases no larger than 0.2 mm as isolated tumour cells or tumour cell clusters; nodes with isolated tumour cells will be classified as node negative (pN0) for stage grouping (3).

One of the most exciting current roles of sentinel lymph node biopsy is the ability to stage patients intra-operatively, allowing a one-step axillary lymph node dissection if the sentinel lymph node contains metastatic carcinoma. Currently, intraoperative evaluation of sentinel lymph nodes is performed using imprint cytology with or without rapid cytokeratin staining, frozen sectioning with or without rapid cytokeratin staining, scrape preparations, or some combination of these techniques (2).

Beach etal (7) performed rapid immunohistochemistry using Dako's EPOS Cytokeratin antibody on air dried frozen sections of sentinel lymph nodes taken at intraoperative consultation. Slides were ready for interpretation within 16 minutes. Results were compared with those of the intraoperative touch preparations and frozen sections and with paraffin-embedded, hematoxylin and eosin-stained, and AE1/AE3 immunostained permanent sections. They found the rapid immunohistochemistry method to be the least sensitive (57% sensitivity) of all methods used to detect metastasis. Routine diagnostic touch preparations, frozen sections, and permanent sections had sensitivities of 69%, 86%, and 100% respectively. They concluded that the rapid immunohistochemistry method would not be helpful in intraoperative assessment of sentinel lymph nodes in breast cancer patients due to its low sensitivity.

The common protocol for the examination of sentinel nodes includes the examination of several H&E levels of the sentinel node. This is often accompanied by cytokeratin staining. Pargaonkar etal (4) undertook a retrospective analysis of 84 breast cancer patients with sentinel node biopsies, who also underwent axillary dissection. They found that Hematoxylin-eosin staining identified 20 patients (23.8%) with sentinel node metastases. The remaining 64 negative patients (76.1%) were tumour free on sentinel lymph nodes at level 1 HE. Additional immunohistochemical stains for keratin and HE stains on specimens from these 64 patients showed an additional 5 patients (7.8%) to be positive for lymph node micrometastases (<2 mm). The total percentage of cases with sentinel lymph node metastases detected by HE staining and immunohistochemistry was 29.7%. Of the remaining 59 cases that were negative on HE and immunohistochemistry, axillary dissection revealed 3 cases that had metastases in the axillary lymph nodes. The false-negative rate was 10.7%. The concordance rate between sentinel lymph nodes and axillary lymph nodes was 96.4%. The sensitivity was 89% and specificity was 100%.

Careful interpretation of cytokeratin immunostaining of sentinel lymph node biopsies, though, is essential, as is awareness of the presence of CK-positive native reticulum cells, to avoid confusion with single cells of metastatic carcinoma. Linden and Zarbo (5) observed that up to 10% of cells in benign, reactive lymph nodes may be immunoreactive with anti- cytokeratin antibodies. AE1/AE3 stained 2 of

20 cases with rare immunoreactive reticulum cells, whereas CAM 5.2 immunostained cells in 85% of cases with reticulum cells in sinuses and the paracortex.

Epithelial inclusions representing ectopic breast tissue are uncommonly seen in axillary lymph nodes. The extensive histopathologic examination of axillary sentinel lymph nodes of patients with breast carcinoma may increase the chances to encounter tiny foci of ectopic breast tissue. This may be misinterpreted as (micro)metastatic disease and lead to unwarranted completion of axillary dissection, to inaccurate staging and improper adjuvant treatments for the patients (6). Maiorano etal (6) reported seven cases of ectopic breast tissue in axillary sentinel lymph nodes. In three cases there were coexistent micrometastases, and in the remaining cases the ectopic tissue was not associated with metastatic disease. The ectopic breast tissue showed remarkably varied morphologic features, including apocrine metaplasia and proliferative changes indistinguishable from those occurring in sclerosing adenosis and florid epithelial hyperplasia of the breast. A peripheral layer of myoepithelial cells was consistently detected in the ectopic glands and ducts. Besides awareness and purely morphologic criteria, a false-positive identification of these inclusions as metastatic carcinoma may be avoided by the use of immunohistochemical reactions for the localization of specific markers of the myoepithelial cell component, which is associated with the ectopic breast tissue (eg smooth muscle actin).

References

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Iron Histochemistry – A Review

It is convenient to divide iron-containing complexes in human tissues into two categories: those in which the iron is loosely bound to proteins and easily released by mild acid treatment (eg hemosiderin) and those in which the iron is more strongly bound (masked iron) and cannot be released by mild acid hydrolysis (eg haemoglobin) (1).

Iron in the body is stored in the forms of hemosiderin (ferric hydroxide polymer) or ferritin (a ferrous iron-protein complex) (1). Iron in tissues occurs mainly in the ferric state (2,3).

The reactions that have been used for the detection of iron in tissues include (2-5):

1. The Quincke reaction using ammonium sulphide
2. The Perls reaction, using ferrocyanide, for ferric and the Turnbull Blue reaction, using ferricyanide for ferrous iron.
3. Coloured lakes, eg haematoxylin (Mallory's Method)
4. Coloured precipitates with organic chemicals not classified as dyes (eg bathophenanthroline).

Ferric iron may be converted into ferrous iron by ammonium sulphide (Quinke's reaction) and the ferrous sulphide thus formed can then be demonstrated using the Turnbull blue reaction (3,5).

Some iron-containing compounds (hemoglobin, malaria pigment, formalin pigment) do not react with the Perl's method because the iron is present in bound form. These compounds can be unmasked using hydrogen peroxide and then demonstrated using the Perl's reaction (1).

Interestingly, it is possible to remove excess iron pigment from tissue sections. Iron can be removed by (5):

- 15 min in 1% sodium dithionite in 0.1M acetate-HCl buffer (pH 4.5)
- 3 hours in 2.4N HCl
- 30 min in 3.7N H_2SO_4
- 15 min in 5% Oxalic acid

Heavily pigmented tissues may need to have these times extended (5).

References

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Abstracts from the literature

The reliability of long-term storage of direct immunofluorescent staining slides at room temperature

Dikicioglu E, Meteoglu I, Okyay P, Culhaci N, Kacar F.,

Journal of Cutaneous Pathology Volume 30: Issue 7 Page range: 430 – 436.

Background:
Long-term preservation of immunofluorescence is important for re-examinations. We investigated whether the storage of direct immunofluorescent (DIF)-positive slides at room temperature was reliable in daily practice.

Methods:
One hundred and twenty-five DIF-positive slides from the skin of 52 patients were evaluated. Sections were examined for the presence of immunoglobulin A (IgA), IgG, IgM, C3, and fibrinogen using fluorescein isothiocyanate-conjugated antisera and mounted with a ready-to-use permanent mounting medium containing an antifading reagent and sodium azide (DAKO, Glostrup, Denmark, S3023). The slides were stored at room temperature for 16–24 months. Changes in diagnostic pattern, fluorescence intensity, and the form and location of accumulation of immunoreactants and technical deformation were investigated.

Results:
Over the entire observation period, 49.6% of the slides faded away; the median length of survival was 16 months. Before 12 months, the survival rate of slides was 92.0%, whereas after 20 months it was 28.0%. In the early faded slides, which faded away before 16 months following the first examination, C3 and IgA were the most frequently observed immunoreactants. The technical deformations did not prevent the diagnosis.

Conclusions:
The preservation of fluorescence in DIF-positive slides using mounting media with an antifading reagent is possible for 2 years at room temperature. However, in daily practice, storage for longer than 11 months prevents a reliable diagnosis.

Masson Trichrome Stain: Postfixation Substitutes

Yufeng Yu and Clifford M. Chapman*

The J Histotechno1., 26:131, 2003

Formaldehyde-fixed paraffin-embedded tissues must be post fixed before staining with the Masson's trichrome method. Although Bouin's solution is specified as the chemical of choice, we report on the results of using different post fixatives in the procedure. The use of Gram's iodine or citrate buffer as a postfixative in the Masson's trichrome procedure produces slides that exhibit superior staining qualities than when Bouin's solution or a Bouin's substitute is used.

Diagnosis of renal amyloidosis using Congo red fluorescence

Sait Sen and Gülçin Basdemir

Pathology International 53(8): 534 - 538

Early diagnosis and classification of amyloid deposition and differentiation from other glomerular fibrillar deposits relies on routine Congo red (CR) histochemistry. Congo red fluorescence (CRF) is an alternative method based on examination of the CR-stained section by ultraviolet (UV) light. The aim of this study is to investigate the usefulness of CRF, especially when applied to frozen kidney sections. Congo red fluorescence was applied to sections of frozen kidney biopsies prospectively and to paraffin sections retrospectively. The findings of CRF were compared to CR staining in bright light. Prospectively, 15 cases of amyloidosis were diagnosed on frozen sections and identical CR staining was found in all of the paraffin-stained sections. There were no false positives or negatives. Retrospectively, 146 renal biopsies previously stained with CR were re-evaluated with CRF. Eighty-seven CR positive cases were confirmed by CRF, and one new case was identified. Congo red fluorescence is simple to perform and more pronounced, therefore easier to evaluate than CR in bright light. Congo red, when combined with immunohistochemistry, is still visible under UV whereas CR is masked in bright light. Although not widely used, the CRF method for detecting amyloid is simple to use with a high specificity and sensitivity, and may be applied successfully to frozen sections.

Fixation Methods for the Study of Lipid Droplets by Immunofluorescence Microscopy

Deanna DiDonato and Dawn L. Brasaemle

J Histochem Cytochem 51:773–780, 2003

The study of proteins associated with lipid droplets in adipocytes and many other cells is a rapidly developing area of inquiry. Although lipid droplets are easily visible by light microscopy, few standardized microscopy methods have been developed. Several methods of chemical fixation have recently been used to preserve cell structure before visualization of lipid droplets by light microscopy. We tested the most commonly used methods to compare the effects of the fixatives on cellular lipid content and lipid droplet structure. Cold methanol fixation has traditionally been used before visualization of cytoskeletal elements. We found this method unacceptable for study of lipid droplets because it extracted the majority of cellular phospholipids and promoted fusion of lipid droplets. Cold acetone fixation is similarly unacceptable because the total cellular lipids are extracted, causing collapse of the shell of lipid droplet-associated proteins. Fixation of cells with paraformaldehyde is the method of choice, because the cells retain their lipid content and lipid droplet structure is unaffected. As more lipid droplet-associated proteins are discovered and studied, it is critical to use appropriate methods to avoid studying artifacts.

Heat-Induced Antigen Retrieval Restores Electrostatic Forces: Prolonging the Antibody Incubation as an Alternative

Thomas Boenisch, M.S.

Applied Immunohistochemistry & Molecular Morphology 2002; 10(4):363-367

The term antigen retrieval was introduced by Shi et al. to describe a process of heating formalin-fixed paraffin-embedded tissue sections for improved immunoreactivity of tissue antigens with their specific antibodies. Although it has currently become an essential part of immunohistochemistry, the exact nature of this process remains unknown. The following report will describe the rationale and results of experiments that associate the restoration of electrostatic (coulombic) forces with the functional basis of antigen retrieval. Critical support for this was derived by the application of relevant tenets regarding the sensitivity of immune reactions to pH and ionic strength and through the use of prolonged antibody incubations. For the majority of the investigated tissue antigens, prolonging the antibody incubation time from a standard 10 minutes to 60 minutes represented an effective alternative to heat-induced antigen retrieval. The report will carefully weigh the advantages and disadvantages of prolonged antibody incubations versus antigen retrieval procedures.

Lung Cancer Patterns Of Care In South Western Sydney, Australia

S K Vinod, G P Delaney, A E Bauman and M B Barton

Thorax 58:690-694, 2003

Cancer Therapy Centre, Liverpool Health Service, Liverpool BC, NSW 1871, Australia
School of Community Medicine, University of New South Wales, Australia
Collaboration for Cancer Outcomes, Research and Evaluation, Liverpool Health Service

Background:
Lung cancer is the leading cause of cancer deaths in New South Wales (NSW). There is a significantly higher incidence of lung cancer in the South Western Sydney Area Health Service (SWSAHS) than the NSW average. The aim of this study was to document patterns of lung cancer care for SWSAHS residents.

Methods:
SWSAHS residents diagnosed with lung cancer in 1993 and 1996 were identified from the NSW Central Cancer Registry and their medical records reviewed.

Results:
The study population comprised 527 patients of median age 68 years. 12% did not see a lung cancer specialist, 9% did not have a pathological diagnosis, and 28% did not receive any active treatment throughout the course of their illness. The median survival was 6.7 months and the 5 year overall survival was 8% (95% CI 6 to 10). The rates of pathological diagnosis, specialist referral, and treatment decreased with older age and poorer performance status.

Conclusions:
The management of lung cancer patients in SWSAHS is suboptimal. A significant proportion of patients are not receiving treatment. To improve patient care and outcomes, all lung cancer patients should be referred to a specialist for management, ideally in a multidisciplinary setting. Both consumers and general practitioners need to be educated about options available for the management of lung cancers and ageist and nihilistic attitudes need to be overcome.

Microbiology Controls – Try Beef Jerky

The weird snippets of information available from the internet is truly amazing. Ross Stapf of the Washington Adventist Hospital (Takoma Park MD) recently responded to Histonet suggesting the use of the "Slim Jim meat snack" – a type of beef jerky, as a wonderful gram positive and negative control.

Producing suitable gram controls is achievable if one has access to a microbiology department. Obtain some fresh placenta from a maternity delivery unit. Ask a microbiologist to inoculate a vial of nutrient broth with a pure culture of the gram-negative bacillus Escherisa coli and gram-positive Staphylococcus aureus. Place slices of placenta into the broth and allow to incubate for 3-4 hours at 37^{o}C. Add 10% formalin to fix and then process as usual.

Steve Scholz suggests the use of orange peel that had began to mould as a wonderful Fungi control. His procedure is as follows:

"Take an orange and let it sit at room temperature until it starts to mould. This may take several weeks so you have to be patient. Transferring mould from some other source to the orange can accelerate the moulding process. A mouldy piece of bread (penicillin) could be used. The bread itself will not stand up to processing. We injected the orange with some mould we found in the pathologist's coffeepot (aspergillus). After the orange is good and mouldy just cut some of the peel off and process on a routine cycle. The peel holds up well. You can then embed, cut and stain like it is a normal piece of tissue."

A veterinary source for a spirochaete control - namely Leptospira has been suggested. It seems that dogs die of leptospirosis fairly often. The organism is somewhat hazardous in the fresh state, but is inactivated by fixation.

Leptospira is a very large spirochaete, probably one of the largest. If it is used as a control, and the patient's tissue has syphilis, one of the smallest spirochaetes, you may end up with a false negative on the patient's tissue.

Spirochetes in general are not easy to culture, and Treponema pallidum, the etiological agent of syphilis, has never been cultured successfully.

Leprosy requires control tissue containing the etiological agent of the disease, Mycobacterium leprae, which has also never been cultured. It is an acid-fast organism, but it does not have the same staining characteristics as Mycobacterium tuberculosis, and there is no substitute for it as a control.

Leprosy affects armadillos, and one leprous armadillo liver would supply a universe of histologists with controls.

Try This Stain

Harada's Oxidative Technique for Mycobacteria

Principle:

This sensitive technique is excellent for demonstrating non-acid fast and weak acid-fast organisms including leprosy bacilli. M. tuberculosis, which may be non-acid fast at some growth stages can be easily demonstrated. This technique though is not specific since several non-acid fast organisms (including corynebacteria, E.coli and propionibacteria) acquire strong acid stable fuchsinophilia after prolonged oxidation.

Fixation: 10% buffered formalin.
Microtomy: Paraffin sections at 5um.

Reagents:

1. 1% Potassium Permanganate
2. 1% Oxalic Acid
3. Strong Carbol Fuchsin Solution (see ZN stain)

   Warning: Suspected Carcinogen, toxic – see MSDS
4. 1% Acid Alcohol (see ZN stain)

   Warning: Flammable – see MSDS

Procedure:

1. Dewax and hydrate sections.
2. Oxidise in 1% Potassium Permanganate, 30 minutes.
3. Wash in water, 3 minutes.
4. Clear with 1% Oxalic Acid, 3 minutes.
5. Wash in water, 3 minutes.
6. Carbol fuchsin 30 min
7. Running tap water Wash well
8. 1% acid alcohol Decolourise sections to pale pink colour
9. Running tap water Wash well
10. Dehydrate, clear and mount.

Results:

Acid Fast Bacilli, leprosy bacilli, corynebacteria, E.coli and propionibacteria	Red

Reference:

Arch.Pathol.Lab.Med., (1987) 111:407-409.

Safety – DPX Hazards

On 20 Mar 2003, David Mehew posted to Histonet:

"Has anyone who uses DPX as a mounting medium noticed that on new bottles and the accompanying hazard data sheets there are two new risk statements? Dibutyl phthalate (the plasticiser used) now carries warnings that state - may cause harm to the unborn child and possible risk of impaired fertility (R61 and R62 in the European system)."

The following information is from the EPA website (http://www.epa.gov/ttnatw01/hlthef/di-n-but.html):

Dibutyl phthalate is used in making flexible plastics that are found in a variety of consumer products. It appears to have relatively low acute (short-term) and chronic (long-term) toxicity. No information is available regarding the effects in humans from inhalation or oral exposure to dibutyl phthalate, and only minimal effects have been noted in animals exposed by inhalation. No studies are available on the reproductive, developmental, or carcinogenic effects of dibutyl phthalate in humans. Animal studies have reported developmental and reproductive effects from oral exposure. EPA has classified dibutyl phthalate as a Group D, not classifiable as to human carcinogenicity.

Dibutyl phthalate is used to help make plastics soft and flexible. It is used in shower curtains, raincoats, food wraps, bowls, car interiors, vinyl fabrics, floor tiles, and other products.

Sources and Potential Exposure

- The largest source of exposure to dibutyl phthalate is from food, possibly fish and seafood; levels in fish ranged from 78 to 200 parts per billion (ppb).
- Dibutyl phthalate levels of 3.3 to 5.7 nanograms per cubic meter (ng/m^3) were detected in the air near New York City. Dibutyl phthalate levels in rooms recently covered with polyvinyl chloride tiles ranged from 15,000 to 26,000 ng/m^3.
- Dibutyl phthalate was detected in some drinking water supplies at levels ranging from 0.1 to 5 ppb

Health Hazard Information

Acute Effects:

- No information is available regarding the acute effects in humans from inhalation or oral exposure to dibutyl phthalate.
- No information is available regarding acute effects in animals from inhalation exposure to dibutyl phthalate, and oral animal studies have reported minimal effects on the liver and a slight decrease in kidney weight.
- Tests involving acute exposure of rats and mice have shown dibutyl phthalate to have moderate toxicity from inhalation exposure and low toxicity from oral exposure.

Chronic Effects (Non-cancer):

- No information is available regarding the chronic effects of dibutyl phthalate from inhalation or oral exposure in humans.
- Limited information is available on the chronic effects of dibutyl phthalate in animals from inhalation exposure; one study reported decreased body weight gain and increased lung weight relative to body weight, and another study reported an increase in brain weight as a percent of body weight.
- Chronic oral studies in animals have reported effects on the liver.

- A 13-week oral study in animals reported reduced body weights, minimal anemia, and liver effects.
- EPA has not established a Reference Concentration for dibutyl phthalate.
- The Reference Dose (RfD) for dibutyl phthalate is 0.1 milligrams per kilogram body weight per day (mg/kg/d) based on increased mortality in rats. The RfD is an estimate (with uncertainty spanning perhaps an order of magnitude) of a daily oral exposure to the human population (including sensitive subgroups) that is likely to be without appreciable risk of deleterious noncancer effects during a lifetime. It is not a direct estimator of risk but rather a reference point to gauge the potential effects. At exposures increasingly greater than the RfD, the potential for adverse health effects increases. Lifetime exposure above the RfD does not imply that an adverse health effect would necessarily occur.
- EPA has low confidence in the study on which the RfD was based because the study used few animals of one sex only and it was not indicated whether the 50 percent mortality observed early in the study was considered treatment-related, nor was the cause of death indicated; low confidence in the database because the study is the only subchronic bioassay of dibutyl phthalate reported in the literature; and, consequently, low confidence in the RfD.

Reproductive/Developmental Effects:

- No studies are available regarding the reproductive or developmental effects of dibutyl phthalate in humans from inhalation or oral exposure.
- Animal studies have reported developmental effects, such as reduced fetal weight, decreased number of viable litters, and birth defects (neural tube defects) in mice exposed orally to dibutyl phthalate. Reproductive effects, such as decreased spermatogenesis and testes weight, have also been reported in oral animal studies.

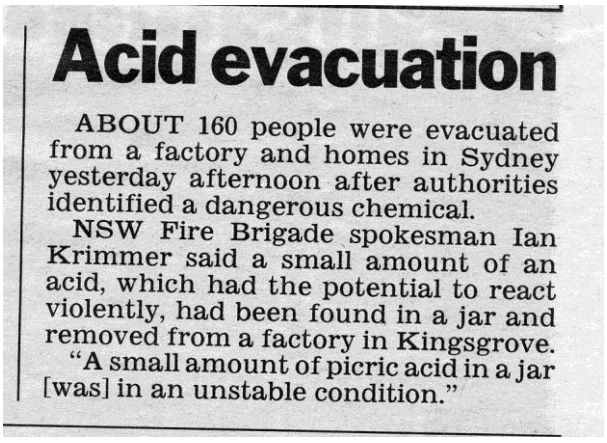
Cancer Risk:

- No information is available on the carcinogenic effects of dibutyl phthalate in humans or animals.
- EPA has classified dibutyl phthalate as a Group D, not classifiable as to human carcinogenicity.

Finally, from Tim Morken:

"The various resins used for coverslip mounting and plastics embedding for both light and EM are all skin sensitisers - some more than others. Generally, the lower the viscosity, the higher the sensitizing hazard."

From the Daily Telegraph 16/9/03, page 3.



Acid evacuation

ABOUT 160 people were evacuated from a factory and homes in Sydney yesterday afternoon after authorities identified a dangerous chemical.

NSW Fire Brigade spokesman Ian Krimmer said a small amount of an acid, which had the potential to react violently, had been found in a jar and removed from a factory in Kingsgrove.

"A small amount of picric acid in a jar [was] in an unstable condition."