Enhanced Histopathology of the Immune System: A Review

Susan A. Elmore, MS, DVM, DACVP, DABT, FIATP
NTP Pathologist and Staff Scientist
National Toxicology Program, NIEHS

IATP Educational Seminar
Uppsala, Sweden
September 7, 2011
Outline

• Historical Perspective
• What is Enhanced Histopathology and Why is it Done, When is it Done, How is it Done?
• The NTP Approach/Experience
• Points to Consider and Caveats
• Thymus and Spleen Examples
Outline

- Historical Perspective
- What is Enhanced Histopathology and Why is it Done, When is it Done, How is it Done?
- The NTP Approach/Experience
- Points to Consider and Caveats
- Thymus and Spleen Examples
Historical Perspective

- **1980**: Immunotoxicity assessment: screening and function studies (Vos)
- **1988**: Development of a testing battery to assess chemical-induced immunotoxicity. NTP’s criteria for immunotoxicity evaluation in mice (Luster et al.)
- **1992**: Risk assessment in immunotoxicology. I. Sensitivity and predictability of immune tests (Luster et al.)
- **1993**: Risk assessment in immunotoxicology. II. Relationships between immune and host resistance tests (Luster et al.)
- **1994**: Histopathology of the immune system as a tool to assess immunotoxicity (Schuurman et al.)
- **1995**: Pathology considerations for, and subsequent risk assessment of, chemicals identified as immunosuppressive in routine toxicology (Basketter et al.)
- **2000**: Histopathologic approaches to detect changes indicative of immunotoxicity (Kuper et al.)
- **2004**: The accuracy of extended histopathology to detect immunotoxic chemicals (Germolec et al.)
- **2004**: Extended histopathology in immunotoxicity testing: interlaboratory validation studies (Germolec et al.)
Guidelines

• Best Practice Guideline for the Routine Pathology Evaluation of the Immune System (STP)

• Harmonization of Immunotoxicity Guidelines in the ICH Process (ESTP)
  – Ruehl-Fehlert et al., *Experimental and Toxicologic Pathology*, 57:1-5, 2005
Three Primary Points

• Each lymphoid organ has separate compartments that support specific immune functions
• These compartments should be evaluated individually
• Semi-quantitative descriptive rather than interpretive terminology
Three Primary Points

• Each lymphoid organ has separate compartments that support specific immune functions

• These compartments should be evaluated individually

• Semi-quantitative descriptive rather than interpretive terminology
Separate Compartments

- Lymph node: cortex (follicles, subcapsular sinus), paracortex (DCU), medulla (cords, sinuses)
- Thymus: cortex, medulla
- Spleen: white pulp (PALS, follicles), marginal zone, red pulp
- MALT: follicles and interfollicular areas
- Bone marrow: various cellular components
• Each lymphoid organ has separate compartments that support specific immune functions

• These compartments should be evaluated individually

• Semi-quantitative descriptive rather than interpretive terminology
<table>
<thead>
<tr>
<th>Interpretative</th>
<th>Descriptive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atrophy</td>
<td>Decreased lymphocytes</td>
</tr>
<tr>
<td>Involution</td>
<td></td>
</tr>
<tr>
<td>Hypoplasia</td>
<td></td>
</tr>
<tr>
<td>Lymphoid depletion</td>
<td></td>
</tr>
<tr>
<td>Hyperplasia</td>
<td>Increased lymphocytes</td>
</tr>
<tr>
<td>Proliferation</td>
<td></td>
</tr>
</tbody>
</table>
Male B6C3F1 Mouse Treated with Citral

Interpretive Terminology: Lymphoid Hyperplasia and Decreased EMH
Descriptive Terminology: Increase in PALS and Marginal Zone Cellularity and Area, Decrease in Red Pulp EMH
Female B6C3F1 Mouse Treated with AZT/Methadone HCL

Interpretive terminology: Atrophy
Descriptive terminology: Decreased area and cellularity of PALS, follicles, germinal centers, marginal zone and red pulp
Outline

• Historical Perspective
• What is Enhanced Histopathology and Why is it Done, When is it Done, How is it Done?
• The NTP Approach/Experience
• Points to Consider and Caveats
• Thymus and Spleen Examples
What is Enhanced Histopathology and Why is Done?

• A tool that the pathologist can use to assist in the identification of immunomodulatory agents

• Allows for the more precise evaluation of cellular changes within lymphoid organs

• Can identify the critical site or compartment within the organ

• Can identify changes in cell production and cell death as well as changes in cellular trafficking and recirculation
What is Enhanced Histopathology and Why is it Done?

- Can provide insight into the putative target cell population and mechanism of action of a test material
- Does not directly measure immune function
When Should Enhanced Histopathology be Performed?

• Specific Immunotox studies
  – A component of the Tier approach

• Subchronic studies
  – Routine histopathology indicates that there may be an immunomodulatory effect
  – Reason to suspect an immunomodulatory effect based on prior information

• Not always recommended for chronic toxicity/carcinogenicity studies
How Should Enhanced Histopathology be Performed?

• Used in conjunction with gross changes, body weights, organ weights (spleen and thymus), clinical pathology
• For immunotox studies, used as a component of a tiered approach that includes non-functional, functional and host-resistance assays
• Semiquantitative evaluation of lymphoid tissue compartments (area, cellularity, cells affected)
• Use descriptive rather than interpretive terminology
How Should Enhanced Histopathology be Performed?

- Evaluate all concurrent controls of a particular lymphoid organ for “range of normal”
- Evaluate same tissues from treatment groups, referring back to control tissues to prevent diagnostic drift
- Repeat control and treatment group evaluations for each set of tissues
- Final interpretations and conclusions should be presented in the pathology narrative
Recommended Lymphoid Tissues for Evaluation

- Thymus
- Spleen
- Bone marrow
- Most proximal draining lymph nodes
- Distal peripheral lymph nodes as a marker for systemic immunomodulation
- Gross lesions
- NTP ITOX Studies: thymus, spleen, bone marrow, submandibular lymph nodes, mesenteric lymph node chain, Peyer’s patches, popliteal lymph nodes, BALT, lung, liver, kidney, adrenals, GI tract
Outline

- Historical Perspective
- What is Enhanced Histopathology and Why is it Done, When is it Done, How is it Done?
- The NTP Approach/Experience
- Points to Consider and Caveats
- Thymus and Spleen Examples
The NTP Approach

– Develop and validate methods to evaluate modulation of immune function
– Evaluate immunomodulatory potential of agents of concern using a tiered testing panel
– Studies to define cellular and molecular events associated with modulation of immune function

Tier I
“Screening Studies”

• Immunopathology
  – Body and organ weights, hematology, splenic cellularity, enhanced histopathology of lymphoid organs and histology of select non-lymphoid organs

• Humoral-mediated immunity
  – Enumerate IgM antibody plaque-forming cells to sheep RBCs

• Cell-mediated immunity
  – Cytotoxic T lymphocytes assay and mixed leukocyte reactivity to allogeneic cells

• Nonspecific immunity
  – Natural killer cell activity

• Cell quantitation
  – Splenic B and T cells
Tier II
“Definitive Studies”

- Repeat immunopathology, humoral mediated immunity and cell quantitation from Tier I studies plus additional tests to assess:
  - Cell mediated immunity
  - Humoral mediated immunity
  - Non-specific immunity
  - Hematopoietic stem cells
  - Host resistance
NTP Experience

- Dibenzanthracene
- Lovastatin
- Genistein
- Black Cohosh
- Diacetyl
- Gum Guggul
- Nelfinavir
- Resveratrol
- Resveratrol NOD
- TCAB
- Sodium Tungstate Dihydrate
NTP Experience

- Either none or subtle changes
- Yet to compare with the non-functional and functional data
- Pathology peer review process is rigorous
- Continuously do the comparison to concurrent controls
- Consider potential processing artifacts including plane of section
Outline

- Historical Perspective
- What is Enhanced Histopathology and Why is it Done, When is it Done, How is it Done?
- The NTP Approach/Experience
- Points to Consider and Caveats
- Thymus and Spleen Examples
Points to Consider and Caveats

• If suspect a test-article effect, compare to organ and body weight, clinical pathology
• The normal histology of some lymphoid organs can be highly variable
  – Mesenteric lymph nodes and Peyer’s patches
  – Consider the range of normal in concurrent controls
• Tissue collection and sectioning is important
  – Collect entire mesenteric lymph node chain
  – Obtain longitudinal sections of lymph nodes
  – Section to obtain largest surface area
  – Superficial/tangential sections may appear as increased/decrease area
  – Section/stain all tissues on same day or randomize samples
Mesenteric Lymph Node Chain

Two Control Thymuses; Variation in Sectioning
Points to Consider and Caveats, Continued

• Specialized techniques such as IHC, blinded review, morphometry, flow cytometry: not to be used as routine screening tools
• Changes in other organs should be considered to determine if there is a secondary effect of the test article
• Distinguish stress/dietary deficiencies/decreased body weight changes from primary test article-related immunotoxic effect
• Severity ranges
Severity Grade Examples

Within normal limits (0)
1-25% minimal (1+)
26-50% mild (2+)
51-75% moderate (3+)
>76% marked (4+)

Within normal limits (0)
1-10% minimal (1+)
11-25% mild (2+)
26-50% moderate (3+)
>50% marked (4+)
How do you know if the identified lesion is due to a primary or secondary effect of treatment?

- Prior knowledge about structure or mechanism of the chemical?
- Changes in concurrent controls?
- Changes in other lymphoid tissues or non-lymphoid tissues?
- Body weight decrease in the treated groups? Is it dose-related?
- Does lesion (such as apoptosis) correlate with decreased body weight?
- Increase in acute phase proteins?
- Are there any animal or environmental stressors?
- What is the nutritional status and overall health of the animals?
- “Weight of evidence” approach
Outline

• Historical Perspective
• What is Enhanced Histopathology and Why is it Done, When is it Done, How is it Done?
• The NTP Approach/Experience
• Points to Consider and Caveats
• Thymus and Spleen Examples
Published Example


- Goal: To use a known immunotoxicant dose regimen of CHB to study the differential toxicity to the lymphoid organs during the atrophic and subsequent recovery phases of drug administration, using the principles of enhanced histopathology to differentiate sensitive from resistant cell populations.

- Groups of treated and control animals were sacrificed regularly during both the treatment and recovery periods.

- Used EH to assess the susceptibility, and recovery, of the different lymphoid cell populations over time.
## Chlorambucil (CHB) Study design

<table>
<thead>
<tr>
<th>Experiment day</th>
<th>17 day treatment period</th>
<th>15 day recovery period</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single i/p CHB (6.3 mg/kg)/vehicle administration</td>
<td>1 3 4 8 11 12 14 17</td>
<td>1 5 9 15</td>
</tr>
<tr>
<td>Necropsy Group</td>
<td>1 2</td>
<td>3 4 5 6</td>
</tr>
<tr>
<td>Day of blood sampling</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Each necropsy group had 5 vehicle controls and 4 treated animals.
### Organ weight changes spleen and thymus

<table>
<thead>
<tr>
<th></th>
<th>Treatment period</th>
<th>Recovery period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 4</td>
<td>Day 12</td>
</tr>
<tr>
<td><strong>Spleen</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relative weight</td>
<td>65.6***</td>
<td>40.7***</td>
</tr>
<tr>
<td>(% of control)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Thymus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relative weight</td>
<td>65.6***</td>
<td>37.2***</td>
</tr>
<tr>
<td>(% of control)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* p< 0.05, ** p< 0.01, *** p< 0.001
Thymic changes during the Treatment period

<table>
<thead>
<tr>
<th>Experiment day</th>
<th>1</th>
<th>3</th>
<th>4</th>
<th>8</th>
<th>11</th>
<th>12</th>
<th>14</th>
<th>17</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose of CHB or vehicle</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>8</td>
<td>11</td>
</tr>
<tr>
<td>Necropsy Group</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

- **Control**
- **Group 1**
- **Group 2**
Thymic changes during the recovery period

<table>
<thead>
<tr>
<th>Dose of CHB or vehicle</th>
<th>1</th>
<th>5</th>
<th>9</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Necropsy Group</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
</tbody>
</table>

Group 3

Group 4
Thymic changes during the recovery period

<table>
<thead>
<tr>
<th>Dose of CHB or vehicle</th>
<th>1</th>
<th>5</th>
<th>9</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Necropsy Group</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
</tbody>
</table>

Group 5

Group 6
Example: Dexamethasone

- Glucocorticoid hormones have their main effect on immature lymphocytes in the cortex
- CD4+CD8+ are most sensitive
- This effect is associated with the relatively abundant expression of glucocorticosteroid hormone receptors
- Expression of the glucocorticoid receptor from the 1A promoter correlates with sensitivity
Sprague Dawley rats treated with 1mg/kg dexamethasone then evaluated at later timepoints.

Control               24 hours         48 hours

12 hours: 3+ increase in apoptotic cells and tingible body macrophages, 3+ decrease in lymphocytes

### Example Checklist for the Evaluation of the Thymus

<table>
<thead>
<tr>
<th>Dexamethasone/Animal #12</th>
<th>↑↓/Severity Grade</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cortex</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Increased/Decreased size</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Increased/Decreased number of small mature lymphocytes</td>
<td>↓3+</td>
<td></td>
</tr>
<tr>
<td>Increased/Decreased number of lymphoblasts</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Increased number of apoptotic cells</td>
<td>↑3+</td>
<td></td>
</tr>
<tr>
<td>Increased number of tingible body macrophages</td>
<td>↑3+</td>
<td></td>
</tr>
<tr>
<td>Necrosis</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td><strong>Medulla</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Increased/Decreased size</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Increased/Decreased number of lymphocytes</td>
<td>↓1+</td>
<td></td>
</tr>
<tr>
<td>Increased number of apoptotic cells</td>
<td>↑1+</td>
<td></td>
</tr>
<tr>
<td>Increased number of tingible body macrophages</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Necrosis</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Prominent epithelial cords and tubules</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Increased Hassall’s corpuscles</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td><strong>Cortex/medulla ratio</strong></td>
<td>Increased/Decreased</td>
<td>X</td>
</tr>
<tr>
<td><strong>Other (give location)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflammation</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Cysts</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Pigment</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Extramedullary hematopoiesis (EMH)</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Comments</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
SA Elmore, Toxicologic Pathology, 34:5, pages 648-655, 2006
<table>
<thead>
<tr>
<th></th>
<th>↑↓/Severity grade</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dexamethasone/Animal #12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Periarteriolar lymphoid sheath</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Increased/decreased size</td>
<td>↓ 3+</td>
<td></td>
</tr>
<tr>
<td>Increased/decreased lymphocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Marginal zone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Increased/decreased size</td>
<td>↓ 2+</td>
<td></td>
</tr>
<tr>
<td>Increased/decreased macrophages</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Follicles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Increased/decreased numbers</td>
<td>↓ 2+</td>
<td></td>
</tr>
<tr>
<td>Increased/decreased lymphocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Increased/decreased germinal centers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red pulp</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Increased/decreased size</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Increased/decreased lymphocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Increased/decreased hematopoietic cells</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Increased numbers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma cells</td>
<td>X</td>
<td>PALS</td>
</tr>
<tr>
<td>Apoptotic cells</td>
<td>↑ 3+</td>
<td>PALS</td>
</tr>
<tr>
<td>Tingible body macrophages</td>
<td>↑ 3+</td>
<td>PALS</td>
</tr>
<tr>
<td>Pigmented macrophages</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Dendritic cells</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Granulocytes/mast cells</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Granuloma/macrophage aggregates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibrosis</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Necrosis</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Comments</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
TCAB perinatal study in SD rats (dosed by gavage on PND 4-21)
Significant dose-related decrease in thymus weights (up to 40%)
Images taken at same magnification
TCAB perinatal study
Images taken at same magnification
Summary

• Appreciation for the dynamic and complex nature of the immune system
• Knowledge of normal structure, function, histology, drainage patterns, species differences
• Standardized sampling and embedding, severity grades
• Evaluate the range of normal changes in concurrent controls first and compare treatment groups to controls
• Evaluate compartments separately for changes in size and cellularity
• Use semiquantitative descriptive rather than interpretive terminology to describe the changes
• Use in conjunction with gross changes, body weights, organ weights (spleen and thymus), clinical pathology, other organ changes, etc.
• Use the pathology narrative for final interpretations and conclusions
• Focus on ensuring consistency in the evaluation and reporting of xenobiotics for immunotoxicity
Thank You!