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NTP IMMUNOTOXICITY STUDY PATHOLOGY SPECIFICATIONS
October 7, 2016

I. Purpose and Background

1. The purpose of the NTP Immunotoxicity Contract is to 1) develop and validate methods to evaluate modulation and pathology of immune function 2) to evaluate the immunomodulatory potential of agents of concern using a tiered testing panel and 3) to use studies to define cellular and molecular events associated with modulation of immune function. The three areas of immune response that are of interest are 1) autoimmunity 2) allergy and 3) immunomodulation.
2. The purpose of the Pathology Specification document is to define the extent of pathology evaluation (necropsy and histopathology), pathology peer review, and archiving of pathology data for the NTP Immunotoxicity studies.
3. The tissues will be obtained from the NTP Immunotoxicology contract laboratory.
4. Specific instructions with regard to the tissues to be collected for histology and other endpoints (such as RNA isolation) will be contained in the NTP Project Approval Committee (PAC) protocol. **The PAC protocol should be considered the primary source of instructions for tissue disposition and may supersede these specifications. The specifications should be considered the default design if no additional information is provided.**
5. Estimated annual number and types of NTP immunotoxicity studies requiring pathology evaluation:

    - Immunomodulation studies
        i. 4 screening studies
            a. Necropsy and histopathology
        ii. 2 definitive studies
            a. Necropsy and histopathology only if the dose is greater than the screening study or requested by the NTP Immunotoxicology Contract COR
        iii. 4 developmental studies
            a. Necropsy and histopathology
    - Hypersensitivity
        i. 4 studies
        ii. Measurements of ear thickness for both ears
        iii. Ear punches at necropsy
        iv. Histopathology of ear punches only if requested by the NTP Immunotoxicology Contract COR
    - Autoimmunity studies
        i. 1 compound
            a. Necropsy and histopathology specific to each study design and dependent on the model selected

6. Study Details

- **Screening studies** are designed to screen for immunomodulatory effects.
    i. One group of vehicle control animals
    ii. Generally 5 treatment groups plus a vehicle control group
    iii. Positive control(s): either cyclophosphamide or dexamethasone
    iv. Each group generally consists of 8 mice or 12 rats, but can vary
    v. Terminal body weights measured for all animals
    vi. All vehicle, test-article treated and positive control animals in the designated assay group shall be necropsied and evaluated for gross lesions and histopathology
    vii. Types of evaluations to be conducted:
        a. Immunopathology – histopathology, clinical pathology, CBC
        b. Evaluation of Innate Immunity – Macrophage function or NK cell assay
        c. Evaluation of Cell – Mediated Immunity – CTL activity
        d. Evaluation of Humoral-Mediated Immunity – Antibody responses to T-dependent antigens
        e. Cell quantification

- **Definitive (immunomodulation) studies** are follow-up studies performed if positive results are detected in the screening studies.
    i. Repeat of positive assays in Screening Studies
    ii. Host Resistance – Assay and endpoint dependent on the identified target in the screening studies
    iii. Necropsy, gross evaluation and histopathology at the request of the NTP Immunotoxicology Contract COR
    iv. Standard design includes only 3 treatment groups with 8-12 animals per group including vehicle and positive control animals
    v. Pathology –only if the dose is different from the screening studies or if requested by the NTP Immunotoxicology Contract COR

- **Developmental immunotoxicology studies** are conducted to assess immunomodulation in animals exposed perinatally to compounds of interest.
    i. Dosing begins on GD 6 and ends on PND 42
    ii. Each group generally consists of 12 animals, usually rats
    iii. Study groups the same as outlined in the screening studies section above

- **Hypersensitivity studies are designed to assess whether compounds of interest induce dermal sensitization.**
    i. Standard design includes 8 mice per treatment group, 3 treatment groups, a vehicle control group and a positive control

ii. Positive control hexyl cimmamal (HCA), isoeugenol or 2,4-dinitrofluorobenzene (DNFB)
iii. Ear thickness will be measured on day 1 prior to dosing, on day 3 2-4 hours following dosing, and on day 6 prior to necropsy
iv. Ear punches will be collected at necropsy

- **Autoimmunity studies** are compound-specific and are designed to assess whether compounds of interest induce or exacerbate disease in autoimmune prone rodent models.
  i. Study design and model selected vary with the compound and putative immune target
  ii. Necropsy, gross evaluation and histopathology at the request of the NTP Immunotoxicology Contract COR

7. Studies are grouped to obtain the maximum information from a given set of animals. All animals in a given study group are euthanized on the same day. Measurement of body weights, organ weights, hematology, gross pathology and histopathology are conducted on a single set of animals.

II. Animals

1. Strains and source: The B6C3F1 (C57BL/6N X C3H/HeN MTV -) hybrid mouse and the Harlan Sprague Dawley rat will be the default animals used in these studies, unless otherwise specified by NTP.
2. Specific mouse models (NZB mouse, NOD mouse, Brown Norway Rat) or strains may be used occasionally at the request of the NTP
3. Balb/C will be used for hypersensitivity studies.

III. Necropsy

1. Animal necropsies shall be performed according to the Mouse Necropsy Procedure for NTP Immunotoxicity Studies (Appendix A).
2. List of tissues to be collected include: spleen, thymus, liver, lung, kidneys, adrenal glands, bone marrow (femur), GI tract (stomach to rectum) with Peyer's patches, mesenteric lymph node chain, mandibular lymph nodes and popliteal lymph nodes. This list of tissues may vary in the PAC protocol, and that protocol should supersede this document.
3. Necropsies shall be done on all found dead or moribund animals that are euthanized early. The tissues shall be stored in formalin and sent to the pathology contract laboratory with the other animal tissues.

IV. Collection of Blood for CBC

1. Refer to Blood Collection protocol (Appendix B).

V. Submission of Pathology Materials From Immunotoxicology Study Contract Lab to NTP and the NTP Pathology Support Contract Lab

1. All tissues will be collected and labeled at the immunotoxicology contract lab. Collected data will be entered into Provantis, generated reports will be uploaded into CEBS, and documents will be stored in CEBS. Wet tissues with animal

identification (sex, strain, age) and a detailed inventory will be shipped/delivered to a contract lab designated by the Contracting Officer's Technical Representative (COR) for NTP pathology support contracts and logged in and inventoried. All study data not directly accessible in the Provantis pathology module shall be uploaded to CEBS once QA has been completed and the COR for NTP pathology support contracts, NTP Archives, the CEBS Scientific Administrator and the NTP Data Collection Unit (DCU) notified via email (Appendix C).

VI. Tissue Processing

1. Tissues will be trimmed and processed (trimming, embedding, sectioning and staining) by the NTP-designated Pathology Support Contract Laboratory according to the specifications in Appendix D. Dates of fixation, ethanol storage and trimming shall be recorded and verified. Slide numbers of slides containing traceable gross lesions (TGLs) shall be recorded in Provantis.
2. Any gross lesions or other observations seen at trimming shall be recorded on the histology processing records and the Pathology Support Contract Lab will notify the COR for the Pathology Support Contract Lab and copy the COR for the Immunotoxicology Contract Lab, DCU and the CEBS Scientific Administrator. A certified exact copy (stamp as a copy and initial/date) of the histology processing record(s) with additional gross lesions will be sent to the Immunotoxicology Contract Lab at the address identified in the laboratory protocol. The additional TGL(s) shall be entered into Provantis by the Immunotoxicology Contract Laboratory staff.
3. The protocol for assigning histology numbers and labeling materials is specified in Appendix E.

VII. Histopathologic Evaluation

1. The initial histology evaluation shall be performed by a contract pathologist (study pathologist) with the guidance of an NTP Pathologist. The COR for the NTP Pathology Support Contract shall assign this study to a contract lab. The contract pathologist assigned to the study shall examine all submitted protocol-required tissues and gross lesions (Appendix A) microscopically for each animal (vehicle and all doses) and render diagnoses, if present. All lesions shall be graded according to the criteria in Appendix F. Positive controls shall be evaluated but not recorded. When possible, it is preferable that the same pathologist conducts the histopathology on all phases of the same chemical being studied. Data shall be entered into the current toxicology data management system (Provantis), the NTP database and acquisition system.
2. A complete histopathologic evaluation shall be performed on early deaths or moribund sacrifices. Immunotoxicology contract lab personnel shall contact the NTP Immunotoxicology Contract COR to determine when histopathology evaluation shall be performed on early deaths. Generally, histopathologic evaluation can be postponed to the end of the study, unless the NTP requests otherwise.
3. The following ancillary data shall be provided in CEBS and considered to aid in histopathological evaluation and interpretation:
   - Laboratory Protocol

- Terminal body weight and organ weight
- Clinical Pathology (CBC) (once available)
- Gross abnormalities report (if any)

The following information or data may be available for some studies and, if so, shall be considered:

- Immunotoxicity report
- In-life data
- Toxicity assay results

4. For all lymphoid organs (thymus, spleen, lymph nodes, BALT, GALT, and bone marrow), the methods of enhanced histopathology as described in the STP position paper: Best Practice Guideline for the Routine Pathology Evaluation of the Immune System (reference 2) and recent publications (references 3-7) shall be employed as directed by NTP. Checklists to use for enhanced histopathology evaluation are provided in Appendix G and, if available, shall be recorded in Provantis. Non-lymphoid organs (liver, kidney, lung, adrenal gland, GI tract) shall have a standard histopathology review performed. Evaluation of tissues with the NTP Pathologist shall be arranged.
5. Ear punches, if deemed appropriate by the NTP Immunotoxicology COR shall be evaluated for changes compared to controls and provided severity scores.
6. An unofficial draft pathology report with checklists shall be prepared and submitted to the NTP Pathologist. After approval, the official draft report with checklists shall be verified to be accurate by the contract laboratory, audited by quality assurance and then be submitted to DCU (electronically and hard copy). After QA by the DCU, and then final approval by the NTP Pathologist, the report is returned to the contract lab for finalization and returned to DCU for distribution.
7. Photodocumentation of representative lesions included in the report shall be acquired according to NTP guidelines (reference 1) and submitted to DCU on CD for storage in the NTP digital image database. DCU will send a copy of the CD to the CEBS Scientific Administrator to be uploaded to CEBS.
8. If a PPR/PWG evaluation by a different pathologist is warranted, then all pathology materials shall be sent to a pathology contract laboratory specified by the COR for the NTP Pathology Support Contract.

VIII. Pathology Review Procedures

1. Overview: Pathology data and reports shall be sent to a pathology contract laboratory for audit of pathology specimens (APS), pathology data review (PDR), and a pathology quality assessment (PQA). These activities shall be performed under the direction of an NTP Pathologist.
2. For each study the following materials will be submitted as part of the pathology data review:
    - Laboratory protocol
    - Carcass (for studies commencing after Oct, 2008)
    - Wet tissues, blocks, and H&E slides
    - Histology processing records
    - Decalcification records
    - Gross abnormalities report (if any)
    - Individual Animal Data (IAD) in Provantis

- Provantis summary tables
- Primary pathology contractor's approved final report
- Body and organ weights
- CBC

3. The following may or may not be received:
    - In-life data
    - Toxicity assays results
4. Reviews:
    - **PDR**: The NTP Pathologist and Study Scientist (NTP Immunotoxicology Contract COR) will review all the available immunotoxicology data and histopathology results. The tissues to be reviewed during the PQA will be determined at the PDR by the study scientist, NTP pathologist, and PDR pathologist.
    - **APS:** An APS shall include a slide-block match, wet tissue review, adherence to NTP immunopathology guidelines, and quality of slide preparation.
    - **PQA:** If the functional assays are negative and the histopathology is negative, and no issues were identified at the PDR, then a Pathology Quality Assessment (PQA) need not be done, unless requested by the NTP. If the functional assays and/or histopathology are positive, A PQA may be performed.
    - **PPR:** The findings from the PQA will determine if resolution of discrepancies requires a Pathology Peer Review (PPR) or convening of a Pathology Working Group (PWG). The PPR would be conducted by the QA Pathologist and organized to include the NTP pathologist, Head of NTP Pathology, Study Pathologist and at least one other pathologist. The Study Scientist or representative will be invited to observe.
    - **PWG:** PWGs are not mandatory for immunotox studies but, if there are major discrepancies or no resolution at the PPR, then a PWG may be convened. Also, if there is a high profile chemical, then a PWG may be conducted in place of a PPR. The PWG will consist of 7-10 pathologists (one or two with immunopathology expertise) and will be organized by the QA pathologist, working closely with the NTP pathologist regarding the issues presented to the PWG panel. After approval by the NTP pathologist, a written report of the findings will be verified by audit at the contract lab and then submitted to NTP for QA and finalizing the Provantis data according to NTP guidelines. NTP will conduct an audit of final data.

IX. Data Entry

1. All diagnoses will be entered into Provantis by the Pathology Support Contract Laboratory.
2. Peer reviewed diagnoses shall be verified by the NTP pathologist and NTP Data Coordination Unit (DCU) staff before being changed and/or made final. Redlining and final review and approval of the pathology data shall be performed by the NTP pathologist and DCU staff.

References
1. Specifications for the conduct of studies to evaluate the toxic and carcinogenic potential of chemical, biological and physical agents in laboratory animals for the National Toxicology Program (NTP), available at http://ntp.niehs.nih.gov.
2. STP Position Paper: Best Practice Guideline for the Routine Pathology Evaluation of the Immune System (2005). P. Haley (chair), R. Perry (cochair), D. Ennulat, S. Frame, C. Johnson, J-M Lapointe, A. Nyska, P. W. Snyder, D. Walker, and G. Walter. *Toxicologic Pathology*, **33**, 404-407.
3. Elmore, S. A. (2006). Enhanced histopathology of mucosa-associated lymphoid tissue. *Toxicol Pathol* **34**, 687-96.
4. Elmore, S. A. (2006). Enhanced histopathology of the bone marrow. *Toxicol Pathol* **34**, 666-86.
5. Elmore, S. A. (2006). Enhanced histopathology of the thymus. *Toxicol Pathol* **34**, 656-65.
6. Elmore, S. A. (2006). Enhanced histopathology of the spleen. *Toxicol Pathol* **34**, 648-55.
7. Elmore, S. A. (2006). Enhanced histopathology of the lymph nodes. *Toxicol Pathol* **34**, 634-47.

Appendix A

RODENT NECROPSY AND TISSUE COLLECTION PROCEDURES FOR NTP IMMUNOTOXICITY STUDIES

1.0 Principle: This procedure allows the evaluation of gross pathology and collection of selected tissues for histopathology for mice. The organ removal and evaluation covered in this appendix includes the spleen, thymus, liver, lung, kidneys, adrenal glands, bone marrow (femur), GI tract (stomach to rectum) with Peyer's patches, mesenteric lymph node chain, submandibular lymph nodes and popliteal lymph nodes.

Organs to be weighed shall include liver, thymus, spleen, right kidney and adrenal gland (separate weights). The lung may be included if it is a target organ.

Specific instructions with regard to the tissues to be collected for histology and other endpoints (such as RNA isolation) will be contained in the NTP Project Approval Committee (PAC) protocol. The PAC protocol should be considered the primary source of instructions for tissue disposition and may supersede these specifications. The specifications should be considered the default design if no additional information is provided.

2.0 Ear thickness measurements and ear punches for hypersensitivity studies

- Ear thickness measurements will be taken for both ears on day 1 prior to dosing, on day 3 2-4 hours following dosing, and on day 6 prior to necropsy, using a calibrated micrometer.

- Histopathology of ear punches will be conducted only if requested by the NTP Immunotoxicology Contract COR. A 6mm ear punch shall be obtained from each ear for each animal following euthanasia. The punches shall be weighed and transferred to a container filled with 10% neutral buffered formalin. Preserved ear punch samples will be transferred to the NTP-approved pathology contractor for histopathology evaluation.

3.0 Necropsy Materials

- Necropsy table with ventilation system
- Examining boards and pins
- CO_2 chamber
- Forceps
- Blunt and surgical scissors
- Single edge razor blades or scalpel
- Specimen containers
- Tissue Cassettes
- Balances and standard weights
- 4 x 4 gauze sponges
- 1 ml disposable syringes
- 23 and 25 gauge disposable needles
- 22-gauge gavage needle
- Suture material
- 10% neutral buffered formalin (NBF), pH 6.8-7.4
- Sheets of dental wax
- Index cards (heavy weight cardstock)
- 100 mm x 15 mm plastic Petri dishes
- Absorbent pads

4.0 Necropsy and Tissue Collection Procedure:

a. Prior to starting necropsy activate the fume recovery system.

b. Each necropsy station should be equipped with its own operating instruments as listed above.

c. All tissue storage containers should be appropriately labeled in advance of the necropsy.

d. Tissue containers should be labeled with the following information Facility/NTP, Study Number, Sex, Animal#, Group

e. All animals should be uniquely identified following AAALAC guidelines and these numbers recorded along with the protocol and study number. Both prosector and recorder together should confirm the animal number with the numbers on the storage containers and record sheets.

f. Accuracy of balances will be determined before weighing mice or organs by weighing standard weights in the range of weights to be evaluated. Record the standard weights and the actual balance readings.

g. Examining boards and instruments must be cleaned with 3% hydrogen peroxide and rinsed in deionized water after each animal necropsy, before the start of another animal necropsy.

h. Tissues will be placed in the appropriately labeled tissue containers containing approximately 200mls of 10% neutral buffered formalin (pH 6.8-7.4). Popliteal and mandibular lymph nodes and adrenal gland(s) will be transferred to cassettes (without foam sponge) that are large enough not to cause tissue impressions and have a mesh size small enough to prevent organs from falling out.

i. Animals are anesthetized by CO_2 inhalation and then removed from the CO_2 chamber for collection of blood for hematology (refer to Appendix B). After blood collection, the animal is returned to the CO_2 chamber for euthanasia and death is confirmed by an AVMA approved secondary method. There should be no other animals in the necropsy room other than the animal being euthanized. The animal should not be placed in the CO_2 chamber until the necropsy team is ready to commence the necropsy upon the animal's death. The chamber must be flushed with room air for 1-2 minutes between each euthanasia to dislodge captured CO_2 from the container. The chamber must also be sanitized after each use to remove fecal material, bedding, etc. The order in which animals are necropsied shall be randomized. Scheduled necropsies and necropsies of moribund sacrifice animals shall be initiated within five minutes after an animal is sacrificed in order to minimize post-mortem autolysis.

j. Record body weight before the animal is given to the prosector. Examine the animal externally and record any gross abnormalities on the record sheet. Spray the animal with 70% ethanol, and wipe the fur with gauze to remove any excess. This will minimize fur contamination of surfaces.

k. Always use a razor blade in cutting tissue to avoid crushing artifact caused by scissors or dull knives. Handle all organs by edges, utilizing connective tissue whenever possible to minimize (eliminate if possible) forcep teeth marks on the organ. When an organ needs to be trimmed, divided or cut, place the organ on a dental wax sheet.

l. Remove a strip of skin from the symphysis pubis to the chin by using forceps and scissors. Use the animal's weight to aid with this cut. Avoid pulling or stretching the skin.

m. Open the abdominal cavity in order to exsanguinate the animal. Puncture the post vena cava and remove as much blood as possible with a 22-gauge needle attached to a 1 ml syringe, using gentle syringe suction.

One may need to rotate the needle if it seems that the bevel is against the vessel wall.

n. Remove the ribcage by making lateral cuts up each side of the ribs through the clavicle. Open the thoracic cavity to expose the heart, lungs, etc. Examine all organs *in situ* prior to removal. Record any abnormal findings. If no gross abnormal findings are observed, the abbreviation “NF” (No Finding) is recorded in the comment section.

o. Record and obtain specimens of all grossly abnormal tissues in addition to those routinely taken.

p. Remove mandibular lymph nodes and place in cassette. The salivary glands may be left attached and removed prior to tissue trimming. Place in the tissue storage container.

q. Remove both lobes of the thymus (without mediastinal lymph nodes attached) as a unit, **weigh** and place on an index card, then place in the tissue storage container.

r. Remove all lung lobes as one pluck (tongue, larynx, trachea, heart and lungs) by grasping the tongue with forceps and cut completely through the jawbone on both sides with scissors. Gently pull the trachea upwards using the forceps, snipping the tissue connections as you go to release the entire thoracic pluck from the body. Remove the tongue, larynx, trachea, heart and lungs (pluck). Insert a gavage needle into the tracheal opening and, using the forceps or a suture to hold the trachea surrounding the needle, infuse the lungs with formalin. For mice, use a 3ml syringe with a 23 to 25-gauge needle and for rats, a 5ml or 10ml syringe with an 18- gauge needle or blunt tip needle if preferred. Be careful not to over-inflate. If forceps were used, keep forceps clamped on the trachea while removing the needle to prevent leakage/backflow of fixative until the suture is secured. If forceps were not used, back the needle out while securing the suture. Tie trachea with suture about midway. Place the inflated lungs into the tissue storage container.

s. Remove the spleen and pancreas as a unit. Separate the pancreas from the spleen. **Weigh the spleen** and place in the tissue storage container. The pancreas is not retained.

t. Remove both adrenal glands and both kidneys. **Weigh right adrenal gland and right kidney** and place both adrenals in a tissue cassette with a "see through" mesh or screen (and without foam sponge). Place in the tissue storage container. A small off center crosswise cut is made in the right kidney to protect the pelvic region. Leave the left kidney uncut. This serves to identify the organs. For rat studies the kidneys should be placed in labeled mega tissue cassettes. Place in the tissue storage container.

u. Remove the liver and **weigh**. Separate the right and left medial lobes with the gall bladder as one unit. Separate the left lateral lobe. Place all lobes in the tissue storage container.

v. When collecting Peyer's patches and the mesenteric lymph node chain, remove the stomach, intestines and rectum as a unit. Gently separate the intestines to expose the mesenteric lymph node chain and its associated adipose tissue. Place the chain of nodes and surrounding tissue (flattened) onto an index card, trim away the intestine and place in the tissue storage container. For the Peyer's patches, infuse the stomach, duodenum, jejunum, ileum, cecum and colon with formalin using a 25-gauge needle and 1 ml syringe. Be careful not to over-inflate the intestines. Place into the tissue storage container.

w. Remove both popliteal lymph nodes from each leg of the animal. These tiny spherical lymph nodes are embedded in adipose tissue of the popliteal fossa and can be collected with the adipose tissue. Place into a tissue cassette with a "see through" mesh or screen (and without foam sponge). Place the cassette into the tissue storage container.

x. Remove the left femur. To remove the femur, a cut is made below the knee joint in the mid portion of the tibia. The knee and this section of the tibia are left attached to the femur. The femur is then separated from the hip joint. Using gauze, remove muscle and tissue from the femur and place in the tissue storage container. If the left femur breaks during collection, the right femur can be collected instead with a comment noted, unless the laboratory protocol designates the right femur for other purposes.

y. Carcasses shall be saved in separate formalin containers once organs are collected.

References

1) Fehlert et al. Revised guides for organ sampling and trimming in rats and mice- Part 1. 2003. 55: 91-106.
2) Kittel et al. Revised guides for organ sampling and trimming in rats and mice- Part 2. 2004. 55: 413-431.
3) Morawietz et al. Revised guides for organ sampling and trimming in rats and mice- Part 3. 2004. 55: 433-449.

APPENDIX B

PROCEDURE FOR BLOOD COLLECTION BY RETRO-ORBITAL BLEEDING FOR NTP IMMUNOTOXICITY STUDIES

1.0 Principle: To collect blood samples from mice or rats for hematology.

2.0 Materials:

- Microhematocrit tubes (non-heparinized) (Fisher)
- Gauze
- 500 μl EDTA microtainer tubes (for HEMATOLOGY, Fisher)
- CO_2 anesthesia chamber

- Procedure:

a. Remove a few microhematocrit tubes from the container, score (with a file) and break each one into 3 or 4 small pieces. This will allow blood to flow quicker and decreases the potential to clot prior to reaching the EDTA tube.

b. Now place the tube in an easy-to-reach location.

c. Place your animal into the CO_2 chamber and open the chamber valve for about 5 seconds to allow gas to enter. Label an EDTA-containing, blood collection tube with the appropriate animal identification number.

d. After applying anesthesia, quickly remove your animal and insert the scored and broken edge of one of your small pieces of the hematocrit tubes into the medial canthus of either eye.

e. Upon inserting the microhematocrit tube, gently twist it about a half turn while at the same time pressing in a downward motion.

f. The moment the tube twists inward, a spontaneous surge of blood flows out. This is why you must have your blood collecting tube in an easy to reach position. Discard the first drop or two to flush the tissue thromboplastin from the microhematocrit tube. Collect an appropriate volume of whole blood in the EDTA-containing, blood collection tube.

g. In most cases, the blood will at some point decrease in flow. This usually indicates that a clot has begun forming within the microhematocrit tube. This is why it is important to have several pieces of microhematocrit tubes broken up prior to bleeding. In less than a few seconds, you can simply remove the clotted microhematocrit tube and quickly insert another into

the medial canthus of the opposite eye (do not resample the same eye). Only collect blood for a CBC analysis (EDTA tube).

h. If a retro-orbital bleed cannot be successfully performed on an animal, then blood collection from the caudal vena cava during necropsy can be done, with a comment noted, and placed into an EDTA-containing blood collection tube.

APPENDIX C

SPECIFICATIONS FOR SUBMISSION OF HISTOPATHOLOGY MATERIALS (WET TISSUES AND PATHOLOGY DATA) FROM THE IMMUNOTOXICOLOGY CONTRACT LAB FOR NTP IMMUNOTOXICITY STUDIES

1. Histopathology materials shall be organized, packed, marked, and delivered or shipped prepaid to the contract lab as directed below.
    a. Prior to shipping materials, the Inventory of Residual Material must be completed. The number and condition of wet tissues shall be shown on this form.

    b. The scheme or SOP used to identify the animals in each study (including an appropriate figure or diagram) shall be submitted at the time that wet tissues are sent to the contract lab.

    c. The NTP Immunotoxicology Contract COR shall provide the appropriate NTP Immunotoxicity experiment numbers that should be used as identifiers for the samples. Each study will have its own unique "I" number.

    d. An email from the Immunotoxicology contract lab indicating intent to ship/deliver showing how many boxes of what kind of histopathology material(s) and on what date(s) shall be directed to the DCU with a copy to the NTP Immunotoxicology Contract COR, the COR for NTP Pathology Support Contract Labs (specifically Mark Cesta), the NTP Archives, and the CEBS Scientific Administrator at least 14 calendar days in advance of shipment/delivery. This is required to aid in tracing lost or misdirected shipments/deliveries, to allow time for the COR to prepare a work assignment, and to notify the pathology support contract lab that the shipment/delivery is expected. The COR for the Pathology Support Contract Lab shall send the work assignment to the Pathology Support Contract Lab and copy the assigned NTP pathologist, DCU, the COR for the Immmunotoxicology Contract, and the CEBS Scientific Administrator.

    e. An electronic copy of inventory and study details (scientific protocol, study design, treatment group identification, clinical observations, body and organ weights, list of tissues collected, clinical pathology, gross pathology, IADs) shall be uploaded to CEBS by the Immunotoxicology Contract Laboratory,

DCU notified, and then DCU shall send electronic copies to NTP Archives, the COR for NTP Pathology Support Contracts, the CEBS Scientific Administrator, and the Pathology Support Contract Lab designated by the COR for NTP pathology support contracts (Appendix C). A hard copy of the detailed inventory shall be submitted with the shipments.

f. For local shipment from the Immunotoxicology Contract Lab to the pathology support contract lab, all wet tissues shall be placed in appropriate containers and double contained to ensure that there is no leakage. Tissues shall be organized by sex, species, strain, age, group, and animal number. Any tissue containing the unique animal identifier (tail tattoo, left ear, etc.) shall be included within the container. Permanent ink shall be used to label the external surface of the containers with the study number, laboratory, group number, and animal number. The boxes shall be marked on one end to show:

Name of contractor Contract Number Experiment number (I #) Animal group number(s) Histology numbers in that box

These tissues will then be shipped/delivered within 4 days following necropsy to a pathology support contract lab designated by the COR for the NTP Pathology Support Contract and in compliance with all local, state and federal regulations.

g. For local shipment from the pathology support contract lab to NTP Archives, all wet tissues (carcasses and residual tissues) shall be placed in two separate heat sealed plastic bags (double bagged), each 4.5 mils thick, one inside the other so that there is no leakage. The tissues shall be organized by sex, species, strain, age, group, and animal number. Any tissue containing the unique animal identifier (tail tattoo, left ear, etc.) shall be included within the container. Permanent ink labels (not ballpoint pen) shall be placed between the two bags showing the study number, laboratory, group number, and animal number. A similar label shall be placed on the external surface of the outer bag. Once the containers or bags are organized, they shall be packed in boxes. The boxes shall be marked on one end to show:

Name of contractor Contract Number Experiment number (I #) Animal group number(s) Histology numbers in that box

h. A hard copy of the detailed inventory shall be shipped with the wet tissues and study details (treatment group identification, clinical observations, body and organ weights, clinical pathology, etc.) shall be available in CEBS.

2. Contact and Shipping Information

- Charles River Laboratories –Pathology Associates
- Contact person
  Dr. Schantel Hayes-Bouknight
  Principal Investigator
  Phone 919-206-7019
  Fax 919-206-7001
  Schantel.Bouknight@crl.com
- Shipping and mailing address
  Charles River Laboratories – Pathology Associates
  Attention Histology
  4025 Stirrup Creek Drive, Suite 150
  Durham, NC 27703

- Integrated Laboratory Systems (ILS)
  - Contact person
    John Pope
    Phone 919-281-1110 ext 835
    Fax 919-281-1118
    jpope@ils-inc.com
  - Mailing address
    ILS, Inc
    P.O. Box 13501
    Research Triangle Park, NC 27709
  - Shipping address
    ILS, Inc
    Attention Histology (John Pope)
    601 Keystone Park Dr., Suite 200, Bay #1
    Durham, NC 27713

c. EPL

- Contact person

Dr. Rodney Miller, Principal Investigator
Phone 919-998-9407 ext. 610
Fax 919-998-9607
miller27@niehs.nih.gov

- Mailing address

P.O. Box 12766
Research Triangle Park, NC 27709

- Shipping address

615 Davis Drive, Suite 500
Durham, NC 27713

d. NTP Archives

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APPENDIX D

TISSUE TRIMMING PROCEDURE FOR NTP IMMUNOTOXICITY STUDIES

1. Tissue trimming shall be supervised by a Pathologist and/or supervisor of the histology laboratory. Their continued presence is not required but they should be available at all times during tissue trimming. The individual animal necropsy findings for each animal shall be available for the technician at the time of tissue trimming. Any additional gross observations identified during the trimming procedure shall be recorded.

2. All gross lesions, and all protocol required organs/tissues shall be trimmed, embedded in paraffin, and H&E stained slides prepared. All early death animals shall be handled in the same manner at the time of trimming.

3 Fixation of tissues as soon as possible after death is preferred for optimal preservation. For screening studies, tissues shall be fixed in 10% NBF then shipped/delivered within 4 days to the assigned pathology support contract lab for immediate processing. The contract lab will be assigned by the COR for the pathology support contracts.

Tissues are to be placed in blocks in a consistent manner so that the same tissues are in the same numbered blocks and placed in the same location within each block for all animals. A general blocking scheme and specific trimming instructions are presented below and are modified for the larger rat tissues. Blocking scheme:

Blocking Scheme for Mice

Block #	Tissue
1	Lungs
2	Liver, left lobe
	Liver, median lobe
3	Kidneys
4	Adrenals
5	Thymus
6	Spleen
7	Mesenteric lymph node chain
8	Stomach
	Cecum
	Colon
	Rectum
9	Duodenum
	Jejunum
	Ileum
10	Popliteal lymph nodes
11	Mandibular lymph nodes
12	Femur

Blocking Scheme for Rats

Block #	Tissue
1a	Right lung lobe
1b	Left lung lobe
2	Liver, left lobe
	Liver, median lobe
3	Kidneys
4	Adrenals
5	Thymus
6	Spleen
7a	Mesenteric lymph node chain
7b	Mesenteric lymph node chain
8a*	Stomach half
8b*	Stomach half
9	Cecum
	Colon
	Rectum
	Duodenum
10	Jejunum
	Ileum
	Popliteal lymph nodes
11	Mandibular lymph nodes
12	Femur

*Cassettes 8a and 8b should each contain a section of the glandular and nonglandular stomach, including the pyloric region.

4. Parenchymal organs, e.g. liver, shall be free of adjacent tissues and trimmed to allow the largest cross-section surface area possible for microscopic examination. Hollow organs shall be trimmed and blocked to allow a cross-section slide from mucosa to serosa.

5. Tissues must be trimmed to a maximum thickness of 0.4 cm for processing. Small (less than 0.4 cm) endocrine organs and lymph nodes shall be submitted intact (whole).

6. Specific trimming instructions:

Spleen: A transverse section shall be taken at the largest extension and **thickest area** of the organ and the cut surface placed down in the cassette. The remaining (longer) section shall be microtomed to the greatest width and the cut surface placed down in the cassette. Large samples can be trimmed on one end to fit in the cassette and to allow placement on the slide.

Thymus: The whole thymus shall be placed in a cassette with the ventral aspect down to facilitate sectioning along the longitudinal axis and **thickest area** of both lobes. This gives a standardized longitudinal section showing all anatomical structures of this organ.

Lymph Nodes:

The mandibular and popliteal lymph nodes and the entire mesenteric lymph node chain (Morawietz et al, 2004) shall be submitted whole (untrimmed) in separate labeled cassettes. Large samples can be trimmed on one end to fit in the cassette and to allow placement on the slide. It is important that a section is taken through the middle of the longitudinal axis of the lymph node at the area of **greatest thickness** in order to avoid a superficial cut and to be able to include the greatest amount of tissue for histological evaluation and thus examine all major areas, such as cortex, paracortex and medulla.

Liver: A transverse section of the left lateral lobe and longitudinal sections of the left and right medial lobes, including the gall bladder (if present), shall be prepared (Ruehl-Fehlert et al, 2003).

Lung: The lung can be separated from the pluck and the extra tissues (tongue, larynx, trachea, heart) discarded. The intact lung of the mouse can be oriented in the cassette to obtain coronal (perpendicular to a sagittal plane and parallel to the long axis of the body) sections of both right and left lungs including main stem bronchi. For the rat, the lobes must be separated and placed in different cassettes, labeled with 1a and 1b, and the same coronal sections obtained.

Kidney: The left kidney shall be sliced along the mid-longitudinal axis and the right kidney shall be sectioned transversely, just off center to preserve the pelvic region, through the entire cortex, medulla and pelvis and one half of each kidney shall be submitted.

Adrenal Glands: Both adrenal glands shall be embedded intact. Sections of adrenal gland shall include the cortex and medulla.

Bone Marrow (femur): After decalcification, the distal 1.5 cm (minimum length for rat) or 1 cm (minimum length for mouse) of the femur must be sectioned through the frontal plane to include the articular cartilage and articular surface, the femoral condyles with epiphyseal plate, and diaphysis with bone marrow. Sections of bone must include the joint surface and marrow cavity. A rapid decalcifier solution such as RDO Rapid Decalcifier (Apex Engineering Products Corp.) can be used for decalcification of the femur following suggested manufacturers recommendations for decalcification of the femurs of rats and mice.

Stomach: The stomach shall be opened along the greater curvature and the mucosa and examined for gross lesions at trimming. The stomach must be cut first through the mid-sagittal plane thereby dividing it into two equal halves and both halves should be submitted for histology. Any gross lesions must also be embedded and sectioned. Sections of the rat stomach shall include the glandular and nonglandular stomach and the pyloric region.

Small and Large Intestines:
One transverse section from each part of the fixed unopened bowel shall be taken according to the localization guidelines provided below. Transverse sections that include all visible Peyer's patches shall be taken. **The remaining intestine shall be opened and examined; all observed gross lesions/abnormalities shall be sampled**

for histopathological examination.

NOTE - *Peyer's patches of the small intestine are mostly visible as slightly elevated lighter nodular areas in the wall of the intestine seen from the serosal surface and are prominent when activated.*

Localizations:

Rat

Duodenum:

- 1 cm distal to the pyloric sphincter in conjunction with a piece of adjacent pancreas.

Jejunum:

- central section containing Peyer's patch.

Ileum:

- 1 cm proximal to cecum.

Cecum:

- central section (but due to larger diameter it is advisable to open section and then take a transverse section).

Colon:

- central section.

Rectum:

- 2 cm proximal to the anus; *optional* longitudinal vertical section to include the anus.

Mouse

Duodenum:

- 0.5 cm distal to the pyloric sphincter in conjunction with a piece of adjacent pancreas.

Jejunum:

- central section containing Peyer's patch.

Ileum:

- 0.5 cm proximal to cecum.

Cecum:

- central section (but due to larger diameter it is advisable to open section and then take a transverse section).

Colon:

- central section.

Rectum:

- 1 cm proximal to the anus; *optional:* longitudinal vertical section to include the anus.

7. All trimmed tissues shall be processed (dehydrated and infiltrated with paraffin) using appropriate chemicals, by an automatic tissue processor, sectioned and stained as indicated in the following sections.

8. After tissues are processed, they shall be embedded in paraffin blocks.

9. Tissues shall be sectioned in organ groups, in a random animal order, and by the same technician to prevent variation in tissue thickness. For example, all spleens shall be sectioned at one time, in random order, and by the same technician. Tissues shall be sectioned at 4-5 microns in thickness. Blocks shall be resealed using a warm spatula to melt the surface wax. Blocks shall be clearly identified with the designated letter code, plus the laboratory's histology number and sub-numbered from 1 through n to indicate the block number for that animal.

10. Tissue slides shall be stained with hematoxylin and eosin, all slides shall be stained on the same day to prevent staining variation, after which they shall be cover-slipped, with glass cover slips. Each slide shall be permanently paper labeled, or labeled using an approved alternate method.

11. All slides, including stained and cover-slipped smears, when required, shall be subject to quality assessment before microscopic evaluation.

12. Slides shall be compared to the blocks (slide-block match-up) to ensure that all embedded tissues are represented on the slide and that the slide number matches the block number.

13. A Histology Processing Record shall be completed for each animal for which histology slides are prepared, and shall be submitted to the NTP with the Individual Animal Necropsy Record. The Histology Processing Record shall include, but is not limited to, the following information:

a. Header information to include test article, histology accession number, species and sex.

b. List of tissues trimmed, number of cassettes prepared and verification by trimming technician with initials and date.

c. List of tissues embedded, number of paraffin blocks prepared and verification by embedding technician with initials and date.

d. Number of blocks sectioned, number of slides prepared and verification by microtomy technician with initials and date.

e. Number of slides stained and cover-slipped with verification by technician with initials and

date.

f. Number of slides checked out during quality control procedure and verification by technician with initials and date.

g. Number of block re-cuts and/or wet tissue re-cuts and verification by technician with initials and date.

h. Notes documenting deviations from protocol, missing tissues, missing gross lesions, problems, and/or comments.

i. Signature of histology laboratory supervisor indicating review and approval of Histology Processing Record.

References:

Morawietz G, Ruehl-Fehlert C, Kittel B, Bube A, Keane K, Halm S, Heuser A, Hellmann J (2004) Revised guides for organ sampling and trimming in rats and mice – Part 3. A joint publication of the RITA and NACAD groups. *Exp Toxic Pathol* 55: 433–449

Ruehl-Fehlert C, Kittel B, Morawietz G, Deslex P, Keenan C, Mahrt C, Notle T, Robinson M, Stuart B and Deschl U (2003) Revised guides for organ sampling and trimming in rats and mice – Part 1. A joint publication of the RITA and NACAD groups. *Exp Toxic Pathol* 55: 91-106

APPENDIX E

ASSIGNING HISTOLOGY NUMBERS AND LABELING CASSETTES AND SLIDES FOR NTP IMMUNOTOXICITY STUDIES

Each study will have a unique NTP I number, assigned by the Immunotoxicology Contract COR. At the time of assignment, this number shall be entered in a permanent log and cross-referenced to the Provantis animal identification number.

- This histology number shall appear on the tissue cassette label (paraffin block), on the slide, and on the label between the two bags containing the wet tissues. This label shall also show the group and animal number.
- The format that shall be used for labeling cassettes and slides is shown below. Each cassette and slide shall be sub-numbered from 1,2,3.....n to show which number that cassette/slide is for that animal.
- The paraffin tissue block (cassette) label and the label between the tissue bags shall use the NTP designated letter code (acronym) for the laboratory and shall precede the histology number.

FORMAT FOR NECROPSY TISSUE CASSETTES

Line 1: Immunotoxicology contract lab designation/NTP and I number

Example for Line 1: BRT/NTP I20045
Line 2: Sex/Individual animal number and tissue
Example for Line 2: F101 Popliteal LN

FORMAT FOR PARAFFIN BLOCK TISSUE CASSETTES
Line 1: Pathology contract lab designation/NTP and I number
Example for Line 1: PAI/NTP I20045
Line 2: Histo # and treatment group/sex/animal number and block #
Example for Line 2: 15-92 AF101 1

FORMAT FOR SLIDE LABELS
Line 1: Immunotoxicology contract lab designation/Pathology contract lab designation/NTP
Line 2: I number
Line 3: 7 Digit Provantis Number
Line 4: Treatment group designation (includes sex) and individual animal number
Line 5: UAR number - Slide number

Treatment/Dose Group Designations
The treatment/dose group designation shall consist of two letters. The first letter shall represent the treatment/dose group and the second letter shall be M for male or F for female. The letter designations for treatment/dose group are as follows:

For all NTP Immunotoxicology Studies
V = Vehicle Control
X = Untreated, Chamber
XP = Positive control
A, B, C, … = Test Article Dose levels in order from lowest to highest

Examples:
CM035 (Third dose male #035)
AF389 (First (Lowest) dose female #389)
EM400 (Fifth dose male #400)

Sample Slide Label:
BRT/ILS/NTP or
BRT/PAI/NTP or
BRT/EPL/NTP
I20045
05921-01
AF101
58731 1

APPENDIX F

GRADING CRITERIA FOR ENHANCED HISTOPATHOLOGY EVALUATION OF NTP IMMUNOTOXICITY STUDIES

In order to best assess and compare the immunomodulatory effects of the various chemicals being evaluated in the NTP immunotox studies, the following grading criteria shall be applied to each lymphoid tissue. These grades refer to relative increases or decreases in cell number, increases or decreases in lymphoid structures (i.e. germinal centers) and increases or decreases in the area of structures (i.e. PALS) per lymphoid organ. Since NTP is looking for subtle changes in lesions we have devised a modified grading scheme. There should be routine evaluation of non-lymphoid tissues.

The grading scheme for the enhanced histopathology evaluation of lymphoid organs is unique and different from that of routine evaluations (Elmore, 2012). Below is a stepwise protocol for the evaluation and grading of these studies.

Step 1: For a given lymphoid tissue, this grading scheme would be applied after first evaluating all concurrent control slides of that one lymphoid organ (i.e. thymus) and determining the "range of normal" with all given a severity score of zero. Outliers in the control group shall be diagnosed and graded but not included for calculating mean severity scores. Those animals shall be excluded from the study and an explanation provided in the pathology narrative.
Step 2: Once a "range of normal" is determined for that group of control slides, the pathologist can pull the two slides that represent the limits of the lowest and the highest range of normal and use these to compare with the tissues from the treated groups.
Step 3: Next the same lymphoid tissues (i.e. thymuses) from the high dose group would be evaluated, carefully comparing to the two "range of normal" slides from the concurrent control.
Step 4: This type of evaluation is done for each of the lymphoid organs (thymus, spleen, lymph nodes, bone marrow, MALT) separately so as not to incur "diagnostic drift".

If a tissue from a treated animal has changes that are considered within normal limits, then it would receive a score of "0". Approximate increases or decreases (%) in cell number, structure area or number would be graded as 1+ to 4+ according to the following criteria:

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

Examples of how to apply the grading criteria:

Spleen, periarteriolar lymphoid sheath

- Increased area, mild (2+) = The area of the PALS is increased 26-50% above the upper range of concurrent controls.
- Increased lymphocytes, minimal (1+) = The number of lymphocytes is increased 1-25% above the upper range of concurrent controls.

Spleen, marginal zone

- Decreased area, marked (4+) = The area of the marginal zone is decreased more than 76% below the lower range of the concurrent controls.
- Increased macrophages, moderate (3+) = The number of macrophages is increased 51-75% above the upper range of concurrent controls.
- Lymphocytes- within normal limits (0) = Cannot be distinguished from concurrent controls

Reference

Elmore, SA (2012). Enhanced histopathology of the immune system: A review and update. Toxicol Pathol 40: 148-156

APPENDIX G

CHECKLISTS FOR ENHANCED HISTOPATHOLOGY EVALUATION OF NTP IMMUNOTOXICITY STUDIES

Spleen: Enhanced Histopathology Checklist

Chemical/Animal #			
	Yes/⇑⇓/Severity grade	No	
Periarteriolar lymphoid sheath			
Increased/decreased area			
Increased/decreased lymphocytes			
Marginal zone			
Increased/decreased area			
Increased/decreased lymphocytes Increased/decreased macrophages			
Follicles			
Increased/decreased numbers			
Increased/decreased lymphocytes			
Increased/decreased germinal centers			
Increased apoptosis			
Increased tingible body macrophages			
Red pulp			
Increased/decreased area			
Increased/decreased lymphocytes			
Increased/decreased hematopoietic cells			
Increased numbers			Location
Plasma cells			
Apoptotic cells			
Tingible body macrophages			
Pigmented macrophages			
Dendritic cells			
Granulocytes/mast cells			
Granuloma/macrophage aggregates			
Fibrosis			
Necrosis			
Other			
Comments			

Thymus: Enhanced Histopathology Checklist

Chemical/Animal #		
	Yes/⇑⇓/Severity grade	No
Cortex		
Increased/Decreased area		
Increased/Decreased number of lymphocytes		
Increased numbers of apoptotic cells		
Increased numbers of tingible body macrophages		
Necrosis		
Medulla		
Increased/Decreased area		
Increased/Decreased number of lymphocytes		
Increased numbers of apoptotic cells		
Increased numbers of tingible body macrophages		
Increased numbers of Hassall's corpuscles		
Necrosis		
Prominent epithelial cords and tubules		
Cortex/medulla ratio		
Increased/Decreased		
Epithelium free areas (EFAs): rats only		
Not evaluated		
Not present		
Increased/Decreased area		
Increased/Decreased number of lymphocytes		
Increased numbers of apoptotic cells		
Increased numbers of tingible body macrophages		
necrosis		
Other (give location)		
Inflammation		
Cysts		
Pigment		
Extramedullary hematopoiesis (EMH)		
Other		
Comments		

Lymph Node: Enhanced Histopathology Checklist (Indicate lymph node location)

Chemical/Animal #		
	Yes/⇑⇓/Severity grade	No
Cortex		
Increased/Decreased area		
Number of follicles		
Increased/Decreased		
Germinal center development		
Increased/Decreased		
Increased/Decreased numbers		
Lymphocytes		
Increased numbers		
Apoptotic cells		
Tingible body macrophages		
Plasma cells		
Pigmented macrophages		
Granulocytes (indicate type)		
Necrosis		
Granuloma/macrophage aggregates		
Interfollicular area (note changes)		
Paracortex		
Increased/Decreased area		
Prominent HEV		
Increased/Decreased numbers		
Lymphocytes		
Increased numbers		
Apoptotic cells		
Tingible body macrophages		
Plasma cells		
Pigmented macrophages		
Granulocytes (indicate type)		
Necrosis		
Granuloma/macrophage aggregates		
Medullary cords		
Increased/Decreased area		
Increased/Decreased numbers		
Lymphocytes		
Macrophages		
Plasma cells		
Increased numbers		
Apoptotic cells		
Tingible body macrophages		
Pigmented macrophages		
Granulocytes (indicate type)		
Necrosis		
Granuloma/macrophage aggregates		
Subcapsular/transverse, medullary sinuses		
Increased numbers		
Lymphocytes		
Macrophages		
Plasma cells		
Pigmented macrophages		

Granulocytes (indicate type)		
Other/Comments		

MALT: Enhanced Histopathology Checklist (specify type: GALT, BALT)

Chemical/Animal #			
	Yes/⇑⇓/Severity grade	No	
Follicles			
Increased/Decreased area or number			
Increased/Decreased numbers of lymphocytes			
Increased/Decreased germinal centers			
Interfollicular area			
Increased/Decreased area			
Increased/Decreased numbers of lymphocytes			
FAE ulceration			
Prominent HEV			
Increased numbers of			Give location
Apoptotic cells			
Tingible body macrophages			
Plasma cells			
Pigmented macrophages			
Granulocytes (type)			
Granuloma/macrophage aggregates			
Necrosis			
Other			
Comments			

Bone Marrow: Enhanced Histopathology Checklist

Chemical/Animal#		
	Yes/⇑⇓/Severity Grade	No
Increased/Decreased number of cells		
Myeloid		
Erythroid		
Myeloid/Erythroid ratio	Ratio =	
Megakaryocytes		
Adipocytes		
Reticular adventitial cells		
Macrophages		
Granulocytes (type)		
Maturation index abnormal?		
Myeloid		
Erythroid		
Hemosiderin		
Increased		
Decreased		
Necrosis		
Hemorrhage		
Fibrosis		
Granuloma		
Neoplasia		
Other		
Comments		