Interpretation of Hematology Data on Toxicology Studies

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Outline

- Introduction
- Preanalytical considerations
- Erythrocytes
- Leukocytes
- Hemostasis
- Case Examples
Preanalytical Variables Affecting Hematology Results coming from the Clinical Pathology Laboratory

BEFORE THE CP LAB
◆ Sex and Age
◆ Supplier
◆ Housing/Bedding
◆ **Diet/Fasting status**
◆ Time of collection
◆ Site of collection
◆ Order of collection
◆ Anesthesia
◆ Anticoagulant
◆ Sample matrix
◆ Previous blood collections
◆ Other study procedures

IN THE CP LAB
◆ **Order of analysis**
◆ Sample handling
  ◆ Freeze/thaw
  ◆ Time prior to analysis
  ◆ Sample volume
◆ **Instrumentation**
◆ Reagents
◆ Quality control procedures
◆ Training of individual
FYI: Stress Pathways
Change in Housing

- Novel caging transiently increases corticosterone in rats

Fasting

◆ Mice – usually not fasted or only short fast (3-5 hours maximum)

◆ Fasted mice do not drink much
  ◆ hemoconcentration, prerenal azotemia

◆ Rats—overnight fast in USA; often no fast in Europe
**Chronic Decreased Food Intake in Rats**

- Rats fed 60% of ad lib amount
- Restricted diet available in morning disrupts diurnal rhythm of ACTH and corticosterone
- Restricted diet offered in evening maintains diurnal rhythm
- Important when pair-feeding rats

Belda et al (2005) Pharmacol, Biochem and Behav 41-6
Example: Severely Restricted Diets

- 14-day study in rats with 4 groups/no test article
  - Group 1: Ad lib fed
  - Group 2: Intake 75% of ad lib fed group
  - Group 3: Intake 50% of ad lib fed group
  - Group 4: Intake 25% of ad lib fed group
- Clinical pathology and histology after 2 weeks of controlled diets

Levin et al ToxPath 21 (1993) 1-14
plus unpublished data
Example: Severely Restricted Diet

- Cell counts dramatically decreased as percent of control values
- Histology changes
  - Groups 2 and 3: decreased cellularity of bone marrow
  - Group 4: bone marrow necrosis
- Not as consistently observed in other species (+/- humans with anorexia)
Other Preanalytical Variables Prior to Blood Collection

- Order of collection
- Transportation of animals
- Amount, rate, and frequency of blood collection
- Anatomical site for blood collection
  - Indwelling catheter vs. venipuncture or other
- Anesthetic used
Order of Sample Collection, Processing, and Analysis

- Always treat control animals exactly the same as treated animals
- **Collect / process / analyze samples without group or time bias**
- Two approaches; both acceptable
  - Random (generate random list)
  - Round robin/stratified/replicate
    - 1\textsuperscript{st} in group 1, 1\textsuperscript{st} in group 2, 1\textsuperscript{st} in group 3, then
    - 2\textsuperscript{nd} in group 1, 2\textsuperscript{nd} in group 2, 2\textsuperscript{nd} in group 3, etc.
Effect of in-house transport on murine plasma corticosterone concentration and blood lymphocyte populations

Carla K. Drozdowicz, VMD; Theresa A. Bowman, DVM; Maria L. Webb, PhD; C. Max Lang, DVM

◆ Mice divided into 3 groups
  ◆ Control (stayed in animal room)
  ◆ Simulated stress (ACTH injection)
  ◆ Traveling mice (cage moved up and down elevator for 15 minutes)

Drozdowicz et al AJVR 51 1841(1990)
Animal Transport: Effect of Cage Movement

- Thymus weights (mg) after 12 minutes of cage movements and elevator rides

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Animal Transport: Effect of Cage Movement

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Anesthetics/Blood Collection in Rats

- **Anesthetics**
  - Isoflurane minimally stressful
  - Generally CO₂ and pentobarbital stressful

- **Stress of blood collection depends on**
  - Method of collection
  - Rate of blood draw
  - Previous blood collection

Blood Collection Volume and Frequency

- Many clinical pathology changes due to too much blood collected
- Always collect **same** amount of blood from **all** animals
- Collect minimum required for experiment, not maximum allowed by IACUC
- IACUC-allowed blood collection volumes affect results
  - Typical maximum allowable blood collection volume as survival procedure governed by IACUCs e.g.:
    - Generally up to 10% of blood volume
    - Can repeat every 2 weeks
    - If collect less, can collect more often (e.g., 5% repeat every week)

Blood Volume Limitations

◆ Mice (e.g. 30 g)
  ◆ Total blood volume: approximately 2 mL
    30 g x 70 mL/kg body weight = 2.1 mL of blood
  ◆ One mouse: collection of blood at necropsy
    Full hematology/limited chems/no coags
    Separate groups of mice for hematology and full chemistry
    Hematology as survival procedure, chemistry at necropsy
    Separate groups of mice for coagulation tests

◆ Rats (e.g. 200 g female rat)
  ◆ Total blood volume—12.4 mL blood
  ◆ 200 g x 62 mL/kg body weight = 12.4 mL of blood
  ◆ Max collection at one time: 12.4 mL x 10% = 1.24 mL
  ◆ One rat
    Full hematology and clinical chemistry as survival procedure
    Serial collections from the same animal
    Coagulation testing at necropsy
Anesthetics/Blood Collection in Rats

- Effect of volume of blood collection on stress hormones
  - 10% of blood volume: minimal effects
  - 12-16% of blood volume: release of epinephrine, norepinephrine, ACTH and corticosterone
  - ≥ 20% of blood volume: maximum stress
    - ACTH ~5x after collection of 25% of blood volume

Blood Collection Requires Skill

- Good sample quality essential for interpretable results
- Collection problems
  - Unskilled phlebotomist
  - Sick, small (knockout or transgenic) or dehydrated mice
- Common sample quality issues
  - Clotted hematology sample
  - Hemolysis
  - Platelet clumps (evaluate blood smear if platelet counts low)
Blood Collection in Dogs and Nonhuman Primates

- Dogs kept in familiar conditions: very little stress
- Primates more stressed than dogs
  - Stress reduced by training, positive rewards, paired housing, and minimization of room disturbances

Effects of Collection Site (mice)

Table 2. Hematological data from blood samples drawn from three different sites.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Tail Blood</th>
<th>Eye Blood</th>
<th>Heart Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelets (×10^3/ul)</td>
<td>882 ± 95</td>
<td>637 ± 56</td>
<td>344 ± 73*</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>53.15 ± 2.27</td>
<td>46.12 ± 1.90*</td>
<td>41.16 ± 1.11*</td>
</tr>
<tr>
<td>RBC (×10^6/ul)</td>
<td>10.94 ± 0.54</td>
<td>10.14 ± 0.42</td>
<td>9.00 ± 0.22*†</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>48.85 ± 1.94</td>
<td>45.5 ± 0.62</td>
<td>45.5 ± 0.33</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>15.5 ± 1.15</td>
<td>14.28 ± 0.59</td>
<td>13.04 ± 0.34</td>
</tr>
</tbody>
</table>

*= p < 0.05 as compared to the tail blood sample; †= p < 0.05 as compared to eye blood samples.

Effects of Previous Collections (mice)

- Order matters
- First collection has higher RBC, WBC, and PLTs

Table 3. Hematological data from tail blood samples drawn either before (first) or after (second) eye samples.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>First</th>
<th>Second</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelets (×10^3/ul)</td>
<td>882 ± 95</td>
<td>563 ± 69*</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>53.15 ± 2.27</td>
<td>44.5 ± 2.29*</td>
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<td>RBC (×10^6/ul)</td>
<td>10.94 ± 0.54</td>
<td>9.65 ± 0.05</td>
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<tr>
<td>MCV (fL)</td>
<td>48.85 ± 1.94</td>
<td>46.15 ± 0.43</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>15.5 ± 1.15</td>
<td>12.84 ± 0.59</td>
</tr>
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* = p < 0.05. 

Nemzek et al (20010 Inflamm. res. 50: 523–527)
Normal Diurnal Corticosterone Pattern Affects Peripheral Leukocyte Counts

Effect of Restraint Stress on Leukocytes (Rats)

- Neutrophils increased slightly with restraint
- Lymphocytes and monocytes (+/-) decreased

Rapid Leukocyte Recovery after Restraint Stress

Effect of Preanalytical Procedures on Erythrocytes

- **Acute stressors**: transiently increased red cell mass and or reticulocytes
- **Example stressors**
  - Transportation stress in dogs (5-7% increase in red cell mass)
  - Exercise in rats and dogs (6x increase in reticulocyte count; 6-10% increase in hematocrit)
  - Nose-only restraint in rats (2x reticulocytes)
- **Chronic stressors**: decreased red cell mass
  - Severe dietary restriction (Levin et al)
  - Severe traumatic injuries
  - Chronic diseases

Effects of Preanalytical Stressors on Leukocytes

- Epinephrine effects: early (within minutes)
  - ↑ neutrophils (mostly mediated by α-adrenergic receptors)
  - ↑ lymphocytes (mediated by β2-adrenergic receptors)
  - Mostly in proportion to circulating cells
    - Demargination: ↑ blood flow, ↓ adhesion)
    - Contributions from spleen and lungs

- Glucocorticoid effects: later (within 30 minutes to few hours)
  - ↑ neutrophils (↑ half-life, distribution into circulating pool)
  - ↓ lymphocytes (Apoptosis, altered trafficking)
  - ↓ eosinophils (generally most specific and sensitive)
  - variable changes in monocytes

Relative Sensitivity/Specificity of Clinical Pathology Parameters to Preanalytical Stressors

- Leukocyte changes more sensitive than RBC, PLT, and clinical chemistry changes
- Detection of stress-related changes
  - Easier to see increased neutrophils in animals with neutrophil predominance
  - Easier to see decreased lymphocytes in animals with lymphocyte predominance
  - Decreased eosinophils more specific for stress than other leukocyte changes
Toxicologic Effects on Blood Cells: Production, Function, Survival

Hematopoiesis

Circulating blood cells
Questions about Hematology Data

◆ **Is it real?** Is it treatment-related?
  ◆ Use concurrent matched (age, sex, housing, etc) control group
  ◆ Use knowledge about variability of parameters, species differences, etc
  ◆ Large animals: pretest data also important
  ◆ Generally reference intervals are not useful

◆ **Is it bad?** If treatment-related, is it adverse?
  ◆ Use concurrent control data
  ◆ Can use appropriate reference intervals to put change into perspective

◆ **Reference intervals**
  ◆ Reference intervals in toxicity studies not equivalent to clinical reference intervals in terms of utility

*Terminology adapted from Bob Hall of Covance Madison
**Hall RL. Lies, damn lies, and reference intervals (or hysterical control values for clinical pathology data) Toxicol Pathol. 1997 25(6):647-9
Specific Guidelines for Interpreting Hematology from Rodents vs. Large Animals

◆ General: rodents vs. larger animals
   ◆ More rodents in each experiment
   ◆ Pretest data not available or not useful (e.g. rats)—rapid growth phase
   ◆ Maturation during experiment changes values
◆ Mice
   ◆ Less consistent than dogs
   ◆ Data from moribund mice often too variable to be useful
   ◆ Good to have at least 10 mice/sex to compare with control
◆ Rats
   ◆ More consistent than dogs
   ◆ Can measure most parameters with survival collections
   ◆ Serial sampling from the same rat
   ◆ Good to have minimum of 5 rats/sex to compare with wild-type/control (better to have more)
Hematology Tests

- Complete blood count*
- Preparation of blood smear (important!)
- Additional appropriate tests

*Complete blood count includes:
- RBC, HGB, HCT, MCV, MCH, MCHC, RDW, +/- retics
- WBC count and absolute differential counts
- PLT +/- MPV
Only Absolute Counts are Relevant

- For example, report and interpret
  - 5000 lymphocytes/uL (not 82% lymphocytes)
  - 320,000 reticulocytes/uL (not 4% reticulocytes)
- Not useful to report relative reticulocyte or differential leukocyte counts
Hematology Instrumentation

- Instruments with animal-specific applications
  - Siemens/Bayer Advia series (2120, 120, Technicon H-1E)
  - Abbott CellDyn series (3500, etc)
  - Sysmex VT-series instruments
- Other instruments sometimes used
- Asian/European names/model numbers of instruments may differ…
Outline

- Introduction
- Preanalytical considerations
- Erythrocytes
- Leukocytes
- Hemostasis
- Case Examples
Outline of Talk: Erythrocytes

- Determination of RBC parameters
- Red cell mass effects
  - Increased RBC mass
  - Decreased RBC mass
    - Hemorrhage
    - Destruction
    - Decreased production
## Hemoglobin Concentration

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<td>HCT</td>
</tr>
<tr>
<td>RBC</td>
<td>MCH</td>
</tr>
<tr>
<td>MCV</td>
<td>MCHC</td>
</tr>
<tr>
<td>RETIC</td>
<td>RDW</td>
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*Hemoglobin is measured after RBCs are lysed:

\[
\text{Hemoglobin} \xrightarrow{\text{K}_3\text{Fe(CN)}_6} \text{MetHb} \xrightarrow{\text{KCN}} \text{Cyanmethemoglobin}
\]
Red Cell Counting/Sizing

- RBCs flow through aperture
- RBCs sized and counted
  - Optical
  - Electrical impedance
Red Cell Counting/Sizing

- Histogram created
- Midpoint = MCV
- Count = RBC

SIZE (fL)

COUNT

MCV
**MCV and Monkey Source (Cynomolgus)**

- Mainland Monkeys (China)
  - 70-80 fL
- Mauritius Monkeys
  - 58-68 fL
- Use one source if possible
- Compare to pretest only
# Red Cell Counting/Sizing

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<td>RDW**</td>
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* Hematocrit = Mean Cell Volume x Red Cell Count

**RDW is a measure of ANISOCYTOSIS

\[
RDW = \frac{\text{Std Dev} \times 100}{\text{mean MCV}}
\]
## Calculated Red Cell Indices

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**MCHC** = Average weight of hemoglobin as a function of total red cell mass (HGB/HCT, or HGB/MCVxRBC)
# Reticulocyte Counting

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*Always analyze reticulocytes when possible*
RBC/Reticulocyte Analysis

670nm Laser Diode

Oxazine 750 RNA Stain

High angle detector (HGB concentration) (5° - 15°)

Absorbance RNA Content

Low angle detector (cell size) (2°-3°)
RBC V/HC

- Macrocytic hypochromic
- Macrocytic Normochromic
- Normocytic hypochromic
- Spherocytes, Schistocytes, Acanthocytes
- Microcytic hypochromic
- Microcytic Normochromic

Red Cell Hemoglobin Concentration (g/dL)

From MHG - Dr. T. Skelton AACC 2001
Normal hemogram

Regeneration

MCV
MCHC
RDW
RETIC

Volume
HGB Conc

Volume
HGB Conc

count
Size (fL)

count
Size (fL)
Species Differences: Reticulocytes

- Rodents vs. larger animals: shorter RBC lifespan, higher retic counts
- RBC lifespan (retic counts)
  - Primates 120 days (27-125 x10^3 cells/µL)
  - Dogs 100-115 days (17-79 x10^3 cells/µL)
  - Rats 45-50 days (135-250 x10^3 cells/µL)
  - Mice 43 days (221-370 x10^3 cells/µL)
- Rodents: more polychromasia and anisocytosis than larger animals
Morphologic Characteristics of Young Red Cells

- Wright’s-Giemsa type stain
  - Polychromasia (bluish)
- New methylene blue stain
  - Stained reticulum (reticulocytes)
- Increased MCV
  - larger
- Decreased MCHC
  - less hemoglobin/volume
Usefulness of RBC parameters in order of value

1. Red cell mass parameters
   Hgb, Hct, and RBC
2. Parameters indicating accelerated erythropoiesis
   Absolute RET and MCV
3. Other supportive parameters
   RDW and MCHC
4. Hardly ever useful
   MCH
Red Cell Mass Parameters

- Red cell mass is estimated by RBC, HGB, and HCT
  - HGB estimates oxygen carrying capacity
  - HCT estimates volume of RBCs as percent of blood volume
  - RBC: evaluated in conjunction with MCV
Change in RBC Counts Without Change in HGB or HCT

- **Increased RBC with microcytosis**
  - Strain differences, Fe deficiency, portosystemic shunts, interference with hemoglobin synthesis

- **Decreased RBC with macrocytosis**
  - Altered nucleic acid synthesis (reverse transcriptase inhibitors, B12 deficiency, FeLV infection)
Outline of Talk: Erythrocytes

- Determination of RBC parameters
- Red cell mass effects
  - Increased RBC mass
  - Decreased RBC mass
    - Hemorrhage
    - Destruction
    - Decreased production
Increased Red Cell Mass

- **Relative increase** in red cell mass: Dehydration
  - Most common cause of increased red cell mass in toxicology studies
  - Loss of water rather than gain in red blood cells (thus relative)

- **Absolute increase** in red cell mass
  - Excess EPO (exogenous or endogenous)
  - Activating mutations of EPO receptor
  - Decreased oxygenation
    - Abnormal hemoglobin (MetHb, etc)
    - Abnormal oxygenation (pulmonary, cardiovascular)
Decreased Red Cell Mass

- Rare: Relative decreased red cell mass
  - Plasma volume expansion
- Very common: Absolute decreased red cell mass
- Use term “Decreased red cell mass” rather than “anemia”

Definitions of anemia
- RBC mass below reference interval (clinical interval)
  - Most relevant in a clinical setting with clinical reference intervals (as opposed to tox reference intervals)
- Decreased oxygen-carrying capacity of blood
Absolute Decreases in RBC Mass

- Hemorrhage (loss from the vasculature)
- Hemolysis (increased destruction; shortened lifespan)
- Decreased production (bone marrow)
Acute Hemorrhage

- Most common: due to excessive blood collection
  - Over-collection: effect of drug in anemic animals
  - Collect consistent and reasonable amount of blood from all groups
- Obvious hemorrhage
- Response
  - Release of RBCs by splenic contraction
  - Associated with ↑ neutrophils and platelets
  - Recovery by 2 weeks in most species
Chronic Hemorrhage

- Usually associated with RBC regeneration in laboratory animals
- Diagnose via clinical signs, rather than specific hematologic changes
- May have occult loss (gastrointestinal, urinary)
- Long term loss may lead to classic iron-deficiency (non-regenerative)
  - Rare in laboratory animal facilities
  - Ulcerated masses (older rodents)
  - Chronic GI diseases
Hemolysis (destruction of RBCs)

- Synonyms for hemolysis
  - increased RBC destruction
  - shortened RBC lifespan
  - increased RBC turnover
**Hemolysis: Characteristics of Extravascular vs. Intravascular**

**Extravascular**
- Slower onset
- No free HGB in plasma or urine
- ±Increased serum bilirubin
- May not affect RBC mass parameters
  - Increased erythroid precursors
  - “compensated”

**Intravascular**
- Acute disease
- Free hemoglobin in plasma (↑MCHC)
- ±Hemoglobinuria
- ±Increased serum bilirubin
- Other morphologic findings

- Mostly see EXTRAVASCULAR hemolysis in toxicity studies
- Primary exception: some IV drugs
Removal of Circulating RBCs

- RBCs removed by splenic macrophages
- Hemoglobin catabolized to protein and heme
- Heme stored and reused for RBC production
Hemolysis: Responses

- Increased production of RBCs
  - Increased reticulocytes and polychromasia
  - Automated retic very sensitive (very useful when available)
  - EMH (↑spleen wt): mouse>rat>other species
- +/- Pigment in spleen, liver, renal tubules
- +/- Hemolysis-related RBC morphology
  - Spherocytes (remodeling)
  - Schistocytes (fragmentation)
  - Heinz bodies (denatured hemoglobin)
Hemolysis: Oxidation Injury

- Two systems prevent oxidative injury
  - Methemoglobin reductase system
    - Methemoglobin: oxidized hemoglobin (non-functional)
    - NADH-MetHb reductase reduces to methemoglobin to reduced hemoglobin (functional)
    - Activity in rodents>>dogs, people
  - Glucose 6-phosphate dehydrogenase (G6PD) system
    - GSH required to scavenge metabolic oxidants in RBC
    - G6PD reduces NADP to NADPH
    - NADPH reduces oxidized glutathione (GSSG) to GSH
Hemolysis: Oxidation Injury

- Evidence of oxidant damage
  - Decreased red cell mass
  - Heinz bodies (denatured hemoglobin)
  - Eccentrocytes
  - Methemoglobin

- If oxidant injury is suspected
  - Prepare new methylene blue stained slides for possible Heinz body enumeration
  - Measure methemoglobin within 30 minutes of blood collection
Heinz Bodies (Cat)

- Wright’s-Giemsa Stain
- New methylene blue Stain
FYI: Hemolysis: Oxidation Injury

The hexose-monophosphate shunt is the sole source of NADPH in RBCs.
FYI: Oxidant Damage to Red Blood Cells

![Diagram of oxidant damage to red blood cells requiring G6PD](image)

Requires G6PD
Species Differences: Regenerative Processes

- Rodents vs. larger animals: shorter RBC lifespan, higher retic counts
- RBC lifespan (retic counts)
  - Primates 120 days (27-125 x 10³ cells/µL)
  - Dogs 100-115 days (17-79 x 10³ cells/µL)
  - Rats 45-50 days (135-250 x 10³ cells/µL)
  - Mice 43 days (221-370 x 10³ cells/µL)
- Reticulocyte response is more exuberant in rodents
- Changes in reticulocyte counts occur faster
Decreased Red Cell Production: Causes

- Decreased erythropoietin action
  - Renal disease
  - Decreased EPO production
  - Abnormal EPO receptors
- Direct bone marrow effect
  - Cytotoxic effects (precursors or stroma)
  - Defects in hemoglobin or nucleic acid synthesis
  - Abnormal maturation/maturation arrest
PROGENITOR CELLS IN BONE MARROW

Stem cells → BFU-E → CFU-E → Erythroid precursors

Erythropoietin

Red-cell mass

Erythropoietin producer

Drop in oxygen in the blood

Kidney (peritubular)

http://www.powerpak.com/CE/ckd/pharmacy/figures.cfm#figure1
Decreased Red Cell Production: Characteristics

- Absent or inadequate reticulocyte response
  - Inappropriate for decreased red cell mass
  - Lack of polychromasia
  - Automated retics extremely useful

- Bone marrow (discussed in detail in other lectures)
  - No visible change at all
  - Abnormal proportions or morphology of red cell precursors (not necessarily…).
Species Differences: Decreased Red Cell Production

- Rodents vs. larger animals: shorter RBC lifespan, higher retic counts
- RBC lifespan (retic counts)
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- Peripheral RBC effects causing bone marrow suppression occur faster in rodents
**Decreased RBC Production**

<table>
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<th></th>
<th>Regen Anemia (all)</th>
<th>Non-regen dog, NHP</th>
<th>Non-regen rodent</th>
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“Anemia of Chronic Disease”

- Most common cause of decreased red cell mass
- Secondary to many underlying processes
  - Inflammation and endocrine most common
  - Also “poor performing” animals
- Cause of ACD
  - ↓ RBC production, ↑ RBC destruction
  - Sequestration of iron by MΦs (role of hepcidin)
“Anemia of chronic disease”

Adapted from Means and Krantz Blood 80 p1639-1647 (1992)

Interpretation of Reticulocyte Responses

- Decreased reticulocytes: *Always inappropriate in the face of decreased red cell mass*
  - Bone marrow effects
  - Effects of other disease processes (Anemia of chronic disease, renal insufficiency)
- Even normal or increased reticulocyte responses may be inadequate
  - Always need to compare to red cell effects
  - Increased retics may be inappropriate for degree of decreased red cell mass
- Species differences need to be considered in evaluating reticulocytes
Outline

- Introduction
- Preanalytical considerations
- Erythrocytes
- Leukocytes
- Hemostasis
- Case Examples
Leukocytes
Counting/Classifying Leukocytes

- Primary Instruments with animal-specific software
  - Siemens/Bayer Technicon/Advia Instruments
  - Abbott CellDyn Instruments
  - Sysmex VT-X instruments
- Most reliable for healthy animals
  - Monocytes, eosinophils, and basophils correlate less with manual counts
- ALWAYS prepare blood smear
Counting/Classifying Leukocytes: Advia
Leukocytes: WBC Counts

- Normal WBC counts: mouse < rat and rabbit < dog and monkey
- Dependent on sampling site
- Counts of blood taken from peripheral sites (saphenous, etc) tend to be higher than counts from central vessels (aorta, vena cava, cardiac, etc)
Species Differences: Lymphocyte/Neutrophil (heterophil) Ratio

- **Lymphocyte > Neutrophils (heterophils)**
  - Young humans, most nonhuman primates, rats, mice, cows (except young), gerbils, guinea pigs, hamsters, fish, some birds

- **Lymphocytes = Neutrophils (heterophils)**
  - Rabbits, most ferrets, some primates

- **Lymphocytes < Neutrophils (heterophils)**
  - Non-young humans, dogs, cats, horses, some ferrets
**FYI: Leukocyte kinetics in health**

<table>
<thead>
<tr>
<th>Pool</th>
<th>Neuts</th>
<th>Lymphs</th>
<th>Monos</th>
<th>Eos</th>
<th>Baso</th>
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<tbody>
<tr>
<td>Marrow Storage</td>
<td>Y</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>Minimal</td>
</tr>
<tr>
<td>Recirculation</td>
<td>N</td>
<td>Y</td>
<td>N</td>
<td>?</td>
<td>N</td>
</tr>
<tr>
<td>Marginal and circulating pool</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>?</td>
</tr>
<tr>
<td>Blood transit time</td>
<td>10 hrs</td>
<td>Hours-years</td>
<td>18-23 hrs</td>
<td>Minutes</td>
<td>6 hrs</td>
</tr>
<tr>
<td>Tissue half-life</td>
<td>1-2 days</td>
<td>Hours-years</td>
<td>Differ-entiate</td>
<td>Unknown</td>
<td>? up to 2 weeks</td>
</tr>
</tbody>
</table>
Changes in WBC Counts

- More subtle in rodents than large animals
- Most effects are qualitatively the same across lab animal species
- Increased leukocytes: causes
  - Redistribution between marginal/circulating pool
  - Increased production (inflammation, neoplasia)
  - Increased retention in peripheral blood (glucocorticoids, adhesion molecules)
- Decreased leukocytes: causes
  - Redistribution between marginal/circulating pool
  - Decreased production (bone marrow effects)
  - Increased egress from blood
Increased Leukocytes:
Response to Excitement and Stress

- Endogenous or pharmacologic
- More pronounced in dogs and monkeys than rodents
- Catecholamines (minutes)
  - Fright or flight response
  - Increased neutrophils and lymphocytes
  - Generally in proportion to circulating cells (demargination)
  - Increased blood flow, decreased adhesion, contributions from spleen and lungs
- Glucocorticoids (hours to days)
  - Over-diagnosed in toxicology studies
  - Increased neutrophils, decreased lymphocytes and eosinophils
  - Variable changes in monocytes
  - Increased half-life, redistribution
  - Apoptosis

Leukocytes: Monkey and Dog

- More difficult to interpret due to small numbers of animals
- Two pretest samples useful
- Very sensitive to confounding effects (other procedures, sample collection, intercurrent diseases, etc)
- Reference ranges can be useful for historical perspective
  - Only appropriate ranges are useful
  - Age, sex, supplier, diet, vehicle, collection, housing, route of administration, etc.
Increased Leukocytes: Inflammation

◆ Rats and mice
  ◆ Increased production of neutrophils, monocytes, and lymphocytes
  ◆ Increased neutrophils may be subtle compared to other species
  ◆ Generally do not observed band neutrophils in response to mild inflammation

◆ Dogs and monkeys
  ◆ Generally increased neutrophils and monocytes
  ◆ Young monkeys: markedly increased lymphocytes (40-80,000/uL)
WBC Morphology: Neutrophils

Compared to dogs, rats and mice have...

- More neutrophil segmentation
- Fewer bands
Neutrophil Morphology during Increased Production

- May see no change, or
- Band neutrophils
- “Toxic” neutrophils
  - Caused by accelerated production
  - Basophilia, vacuolation, Döhle bodies, toxic granulation
- Ring-form nuclei (rodents, other lab animals)
- Comments re neutrophils apply to heterophils
  - Functionally similar to neutrophils
  - Pink-staining granules (rabbits, Guinea pigs, hamsters)
WBC Morphology: Lymphocytes

- Mostly small lymphocytes
- Some atypical lymphs
- Kurloff’s bodies in some species (guinea pigs)
**Increased Lymphocytes**

- **Inflammation**
  - Prominent in rodents, occasionally with antigenic stimulation in other species (eg monkeys)
  - Persistent lymphocytosis (usually viral)
- **Altered traffic patterns**
- **Demargination (excitement--epinephrine)**
  - Associated with increased neutrophils
  - Lymphocyte effects may be more prominent in species with few neutrophils
- **Increased production**
  - Antigenic stimulation
  - Neoplasia
Decreased Lymphocytes

- Drugs directly affecting lymphocytes (other lectures)
- Glucocorticoid-related
  - Redistribution of lymphocytes
  - May not see neutrophil effect in lab animal species with lymphocyte predominance
  - Lympholysis
- Interference with lymph circulation
- Infection
WBC Morphology: Monocytes

- Similar morphology across different species

Dog

Rat
Monocyte Morphology

- Largest leukocyte on peripheral smears
- Irregular cytoplasmic border
- Gray-blue cytoplasm +/- fine granulation
- Pleomorphic nucleus
Monocyte Counts

- **Increased**
  - Corticosteroids (dogs)
  - Inflammation (inconsistent response)
  - Recovery from inflammation

- **Decreased**
  - Not clinically recognized
  - Observed in conjunction with other cytopenias
WBC Morphology: Eosinophils

Compared to dogs, rats and mice have…

- Band nucleus
- Finer granules that fill the cytoplasm
Eosinophil Counts

- Increased
  - Almost always mediated by release of IL-5 from T-lymphocytes
    - Inflammatory conditions, hypersensitivities, parasitism (NHPs)
    - Chronic inflammation of tissues rich in mast cells (skin, lung, GI, uterus)
  - Blocked egress
  - Increased production (G-CSF)
  - Mouse platelet clumps
- Decreased (automated cell counters)
  - Corticosteroids
**WBC Morphology: Basophils**

Compared to dogs, mice and rats have…
- Very few basophils
- Mice DO have basos
**Basophil Counts**

- **Increased basophils**
  - Allergy, Parasites
  - Associated with increased eosinophils
- **Decreased basophils**
  - Not clinically recognized in most species
  - Pharmacologic administration of corticosteroids
Review: Leukocyte Counts

◆ Normal WBC counts:
  ◆ mouse < rat and rabbit < dog and monkey
◆ Dependent on sampling site
  ◆ Central counts usually lower than peripheral counts
◆ Rodent WBCs
  ◆ Mostly lymphocytes
  ◆ Fewer neutrophils; mouse < rat
  ◆ Very few monocytes, eosinophils, and basophils
Review: Increased Leukocytes

- Endogenous substances (catecholamines, glucocorticoids)
- Altered transit patterns
- Shift from marginal pool
- Increased production
- Decreased egress
Review: Decreased Leukocytes

- Decreased bone marrow production
- Glucocorticoid effects
- Peripheral destruction
- Peripheral demand > bone marrow production
- Cytolysis
IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, IL-15, IL-18, TNF, IFN, m-CSF, G-CSF, GM-CSF, MPC-1, TGF-alpha, TGF-beta, site of collection, chemokines, epinephrine, glucocorticoids …
Outline

◆ Introduction
◆ Preanalytical considerations
◆ Erythrocytes
◆ Leukocytes
◆ Hemostasis
◆ Case Examples
Causes and Symptoms of Hemorrhage

◆ Components of Hemostasis
  ◆ Vessels - injury or abnormalities
  ◆ Platelets - number (thrombocytopenia) and/or function
  ◆ Clotting factors - absence, abnormality, or inhibition

◆ Hemorrhage due to disorders of primary hemostasis (vessels and platelets)
  ◆ From mucosal surfaces (epistaxis, melena, hematuria),
  ◆ Petechial or ecchymotic hemorrhages,
  ◆ Prolonged bleeding after venipuncture or wounds

◆ Hemorrhage due to disorders of secondary hemostasis (formation of fibrin)
  ◆ Into joints
  ◆ Into body cavities
Platelets
Platelet counting/sizing (analogous to RBCs)

- Platelet count
- MPV
- Platelet “crit”
- PDW

Diagram:
- Platelets
- Normal
- Microcytic RBCs
- Giant Platelets
Platelets

- Lifespan varies from 3 to 10 days
- Platelet production regulated by platelet and megakaryocyte mass NOT by platelet number!
Changes in Platelet Size

- Large platelets
  - Observed during accelerated production of platelets
- Mean platelet volume (MPV)
  - Follow on an individual animal basis
  - Compare to pretest values as well as concurrent controls
  - Increased with accelerated platelet production
Platelets

- Platelet counts: rodent >>> non-rodent
- Large platelets more common in rodents
- Increased platelets (sometimes giant platelets)
  - Hemolysis / increased hematopoiesis
  - Also endogenous/exogenous EPO, TPO, other growth factors
- Decreased platelets
  - Platelet clumps; poor technique or sick animal
  - Decreased production, increased consumption or destruction
Decreased Platelets

- Poor sampling technique or difficult collection (sick animal) resulting in PLT clumps
  - Invalidates PLT and MPV
  - **Estimate** from blood smear
- Check WBC parameters
  - PLT clumps counted as EOS by Advia 120
  - ≥4% EOS: do manual count
Coagulation Parameters

Prothrombin Time,
Activated Partial Thromboplastin Time
FYI: Coagulation Parameters

The Physiologic Cascade

Vessel or Tissue Injury

Factor VIIa-Tissue Factor complex

Factor VII

Tissue Factor

Factor IX

Factor IXa

Factor VIIIa

Factor VIII

Factor X

Factor Xa

Prothrombin

Thrombin

Fibrinogen

Fibrin

Fibrin crosslinked

Factor XI

Factor XIa

Factor XII

Factor XIIIa

Factor XIII

Measured by PT

Measured by APTT

http://www.medinfo.ufl.edu/year2/coag/physcasc.html
PT and APTT: Species Differences

- APTT and PT variable across species

<table>
<thead>
<tr>
<th>Test (sec)</th>
<th>Human</th>
<th>NHP</th>
<th>Dog</th>
<th>Rat</th>
<th>Mouse</th>
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<tbody>
<tr>
<td>PT</td>
<td>10-12</td>
<td>10-12.1</td>
<td>8.3-10.0</td>
<td>15.1-16.6</td>
<td>11-15</td>
</tr>
<tr>
<td>APTT</td>
<td>30-45</td>
<td>15-25</td>
<td>11.5-14.5</td>
<td>12.6-16.3</td>
<td>32-50</td>
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- Some instruments invalid for animals
- Beagle dogs: Factor VII Deficiency
Data from Veterinary Clinical Patients

- Tests are not optimized for laboratory animals
- In general, reduction in coagulation factors to <30% of normal needed for prolongation of PT or APTT
- Dogs with clinical bleeding may have only 2-3 sec prolongation in PT or APTT
Interpretation of Coagulation Tests

- Shortened times probably not clinically relevant
- Most common reasons for prolonged clotting times
  - Poor collection (concurrent increase in PT and APTT, and decreased fibrinogen)
  - Poor animal health (sometimes resulting in poor collection)
- Treatment-related
  - General poor health or specific mechanism?
  - Clinical relevance
Outline

- Introduction
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- Hemostasis
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Predictability of Animal Testing

- Predictability of data from preclinical studies for human hematotoxicity $\geq 90\%$ (Olson et al. 2000)
  - Fairly good for drugs that affect conserved processes (e.g. effects on nucleic acid or hemoglobin synthesis)
  - Not as good for drugs that affect processes with species-specific quirks (e.g. hepatic metabolism, receptor-mediated effects, blood cell metabolic pathways)
  - Poor for idiosyncratic drug reactions
Predictability of Animal Testing

- Why are animals studies poorly predictive for idiosyncratic reactions?
  - Relatively few animals tested compared to humans exposed in clinical trials
  - May be idiosyncratic only in one species
  - May be idiosyncratic in all species

Examples

◆ Cross-species predictable toxicities
  ◆ Decreased red cell mass and increased MCV with anti-retroviral anti-nucleosides (AZT, d4T)
  ◆ Anti-cancer effects on proliferating cells

◆ Species-specific predictable toxicities
  ◆ L-sorbose and onions
  ◆ Estrogen toxicity

◆ Human idiosyncratic reactions
  ◆ Chloramphenicol toxicity
  ◆ Potentiated sulfa drugs
Hematotoxicity of L-sorbose and Onions

- **L-sorbose induced hemolysis: dogs>> other species:**
  - Dogs metabolize l-sorbose to sorbose-1-P
  - Sorbose-1-P inhibits hexokinase (1st step in glycolysis)
  - Sorbose not metabolized by RBCs of humans/other species

- **Onion/garlic hemolysis: humans < other species:**
  - Dogs: Chinese dumplings; Cats: baby food; cows and sheep: silage or forage
  - Related to sulfide and sulfates
Hematotoxicity of Estrogen

◆ Dogs >> Rats and mice
  ◆ Rats and mice
    ◆ +/- effects on peripheral RBC and WBC counts
    ◆ Mild decrease in bone marrow stem cells
    ◆ High doses suppress EPO production
  ◆ Dogs
    ◆ Toxicity occurs at physiologically relevant doses
    ◆ Myeloid hyperplasia followed by hypoplasia in all cell lines
    ◆ May be due to species-specific endocrine interactions
◆ Humans
  ◆ No estrogen toxicity identified
  ◆ “The predictive value of [preclinical] estrogen toxicity tests for humans has been disappointing”

Human Idiosyncratic Drug Reactions - Hematotoxicity

- Thrombocytopenia most common
- Agranulocytosis
- Hemolytic and aplastic anemia
- Certain classes of drugs overrepresented

Utrecht AAPS J 7 (2006) E914-21
Human Idiosyncratic Drug Reactions - Hematotoxicity

- **Aplasia**: gold, phenylbutazone, chloramphenicol, penicillamine, NSAIDs, sulfonamides, antithyroid drugs, etc.
- **Agranulocytosis**: antithyroid drugs, phenylbutazone, analgesics, NSAIDs; antipsychotic, hypnosedatives, and antidepressants; anti-epileptic drugs; cardiovascular drugs; anti-infective agents; etc
- **Thrombocytopenia**: gold, heparin, quinine/quinidine, sulfonamides, anticonvulsants, NSAIDs, diuretics, etc
- **Hemolytic anemia**: Beta-lactams, quinine/quinidine, thiazides, sulfonamides, NSAIDs, rifampicin, phenothiazines, etc

Utrecht AAPS J 7 (2006) E914-21
Human Idiosyncratic Drug Reactions - Hematotoxicity

- Small percentage of total number of cases, but large proportion of fatal outcomes
  - Total cases of hematologic IDR
  - Percent of fatal outcomes due to IDR
- Require extensive monitoring for some drugs
  - Early detection of toxicity and prevention of mortality
- Pathogenesis
  - Reactive metabolite formation >> protein adducts >> hapten >> T-cell proliferation
**Human Idiosyncratic Drug Reactions - Hematotoxicity**

- Slow progress/conflicting data on predicting predisposition to idiosyncratic reactions
- Extensive research to find animal models
  - Mostly disappointing results
  - Generally models only replicate part of syndrome
    - Erythroid toxicity without leukocyte or megakaryocyte/platelet toxicity
    - Immune reaction with tolerance and not toxicity
Hematotoxicity of Chloramphenicol

◆ Chloramphenicol used in human medicine in a small number of countries

◆ Three types of human hematotoxicity (50 mg/kg/day)
  ◆ Mild anemia with reticulocytopenia, +/- mild leukopenia and thrombocytopenia
    ◆ Dose-related, occurs during treatment, reversible
    ◆ Associated with bone marrow morphologic changes
  ◆ Aplastic anemia
    ◆ Not dose-related, develops after treatment, irreversible, mortality in past was about 50%
    ◆ Where chloramphenicol marketed
      ◆ <0.01% of patients develop aplastic anemia
      ◆ Roughly half of aplastic anemia cases caused by chloramphenicol
  ◆ Leukemia
    ◆ Occurs in patients surviving aplastic anemia
    ◆ Develops within 6-10 years in 15-19% of aplastic anemia patients
Hematotoxicity of Chloramphenicol

- Spectrum of human toxicity (aplastic anemia, leukemia) not well-predicted by dog or rodent toxicity despite many attempts
  - Only mild dose-related effects on red cell production despite massive doses of drug

- Animal toxicity
  - Chloramphenicol in veterinary companion animal medicine
    - Dogs--not reported to be toxic
    - Cats may develop hematologic plus other toxicities (CNS, etc) at 50 mg/kg/day
  - Rodent studies (mouse, rat, Guinea pig)
    - Evidence of mild decreases in red cell production only
    - After ~ 2 wks of dosing at very high mg/kg/day doses (rat: 3600 to 4000; mouse: 1700; Guinea pig 825)
Hematotoxicity of Potentiated Sulfonamides (broad spectrum antimicrobials)

- Sulfonamide e.g. sulfamethoxazole, sulfadiazine, or sulfadimethoxine)
  - Potentiated with either trimethoprim or ormetoprim
  - Non-potentiated sulfonamides not toxic
- Dose-dependent (non-idiosyncratic) hematotoxicity after prolonged treatment: non-regenerative anemia
- Idiosyncratic reactions
  - Humans
    - Agranulocytosis along with other non-hematologic toxicities
  - Dogs: occurs 5-36 days after initiation of Tx
    - Many manifestations of toxicity
    - Hematologic: thrombocytopenia, neutropenia, hemolytic anemia
      - ~30-40% fatal if thrombocytopenia
  - Same incidence (~0.25%) for dogs and humans
  - Mostly large breeds affected; canine syndrome not useful for research into mechanism
Summary

- Results of hematology tests are sensitive to preanalytical effects. Consistent in preanalytical procedures essential for generation of interpretable results.
- Hematologic toxicities can be assessed by classifying the process and then determining the underlying cause.
- Understanding species differences in hematologic processes is essential for proper interpretation.
- Toxicology studies best predict species-independent hematologic toxicities.
- Idiosyncratic human hematologic toxicities remain poorly understood and poorly represented by animal models.