Interpretation of Hematology Data on Toxicology Studies

Nancy Everds, DVM
Diplomate, ACVP
Amgen, Inc (Seattle, WA)
neverds@amgen.com

Outline

- Introduction
- Preanalytical considerations
- Erythrocytes
- Leukocytes
- Hemostasis
- Case Examples

Preanalytical Variables Affecting Hematology Results coming from the Clinical Pathology Laboratory

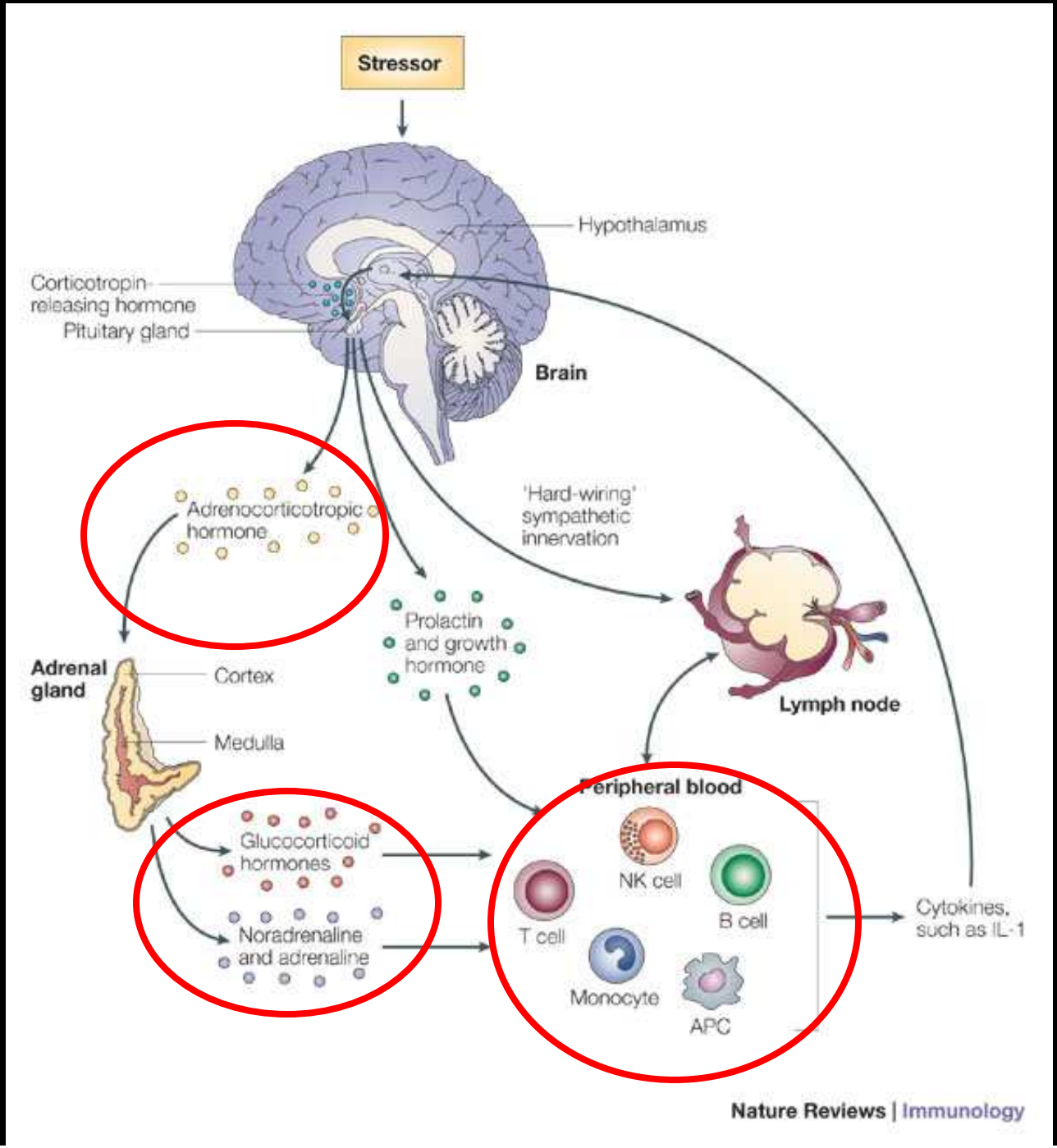
BEFORE THE CP LAB

- 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

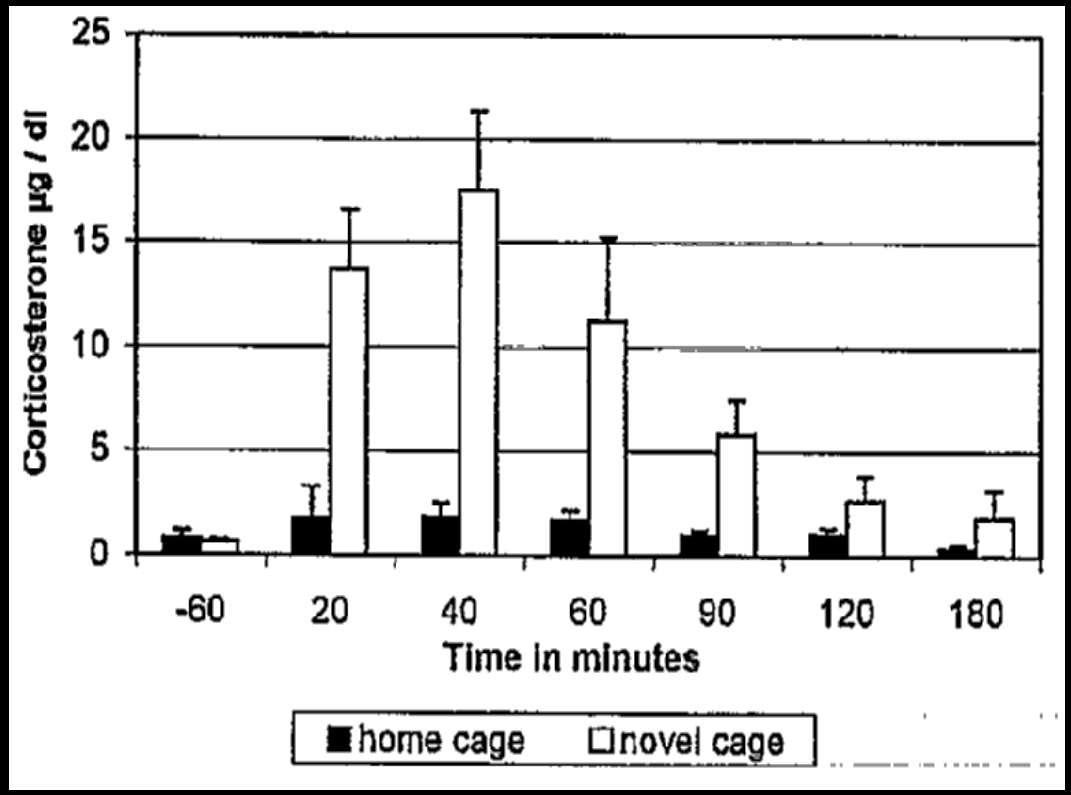
- **Order of analysis**
- Sample handling
  - Freeze/thaw
  - Time prior to analysis
  - Sample volume
- **Instrumentation**
- Reagents
- Quality control procedures
- Training of individual

FYI: Stress Pathways



Change in Housing

- Novel caging transiently increases corticosterone in rats



Fluttert et al (2000) Laboratory Animals 34: 372-78

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
Armario et al (1987) Ann Nutr Metab 31: 81-7
Gallo and Weinberg (1981) J Nutr 111: 208-218

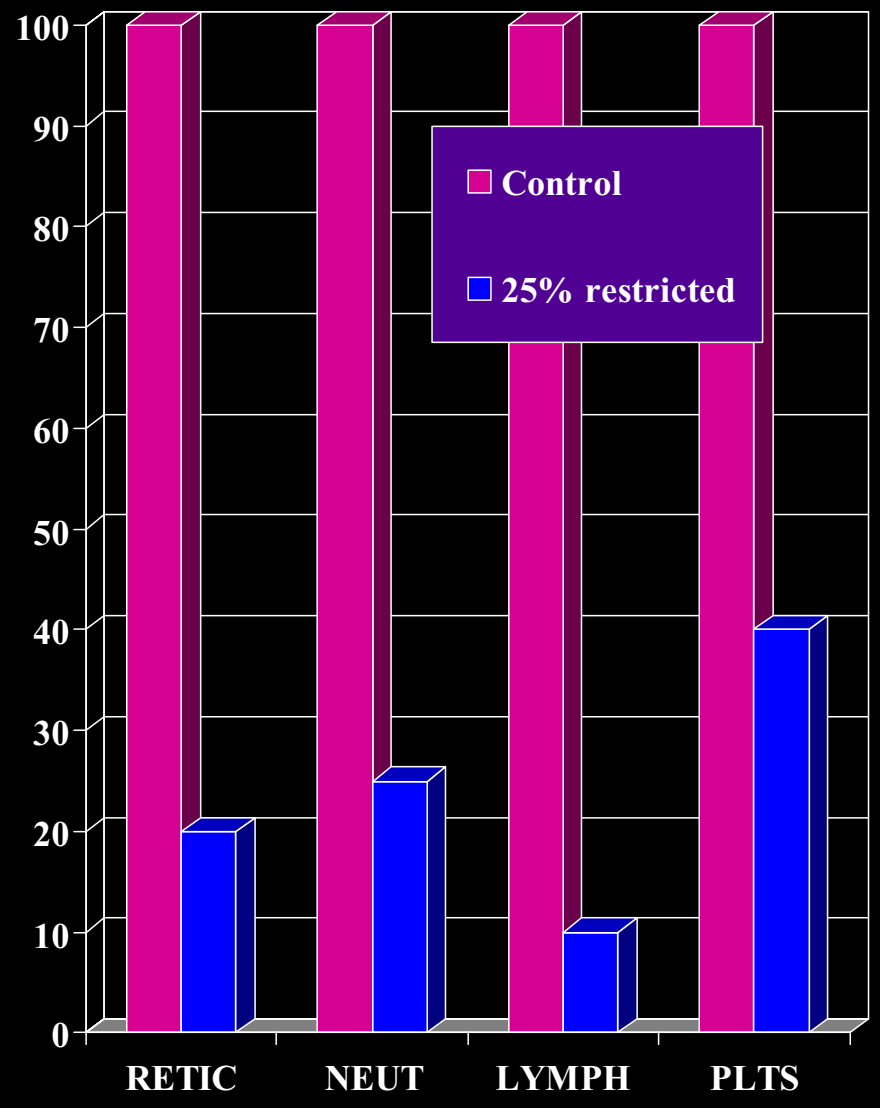
Example: Severely Restricted Diets

- 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
    - 1st in group 1, 1st in group 2, 1st in group 3, then
    - 2nd in group 1, 2nd in group 2, 2nd in group 3, etc.

Animal Transport: Effect of Cage Movement

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

Hrs after Transport	Control	ACTH	Transport
0.25	33.7	33.2	33.6

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

Hrs after Transport	Control	ACTH	Transport
0.25	33.7	33.2	33.6
12	33.0	***28.4***	***29.0***

Drozdowicz et al AJVR 51 1841(1990)

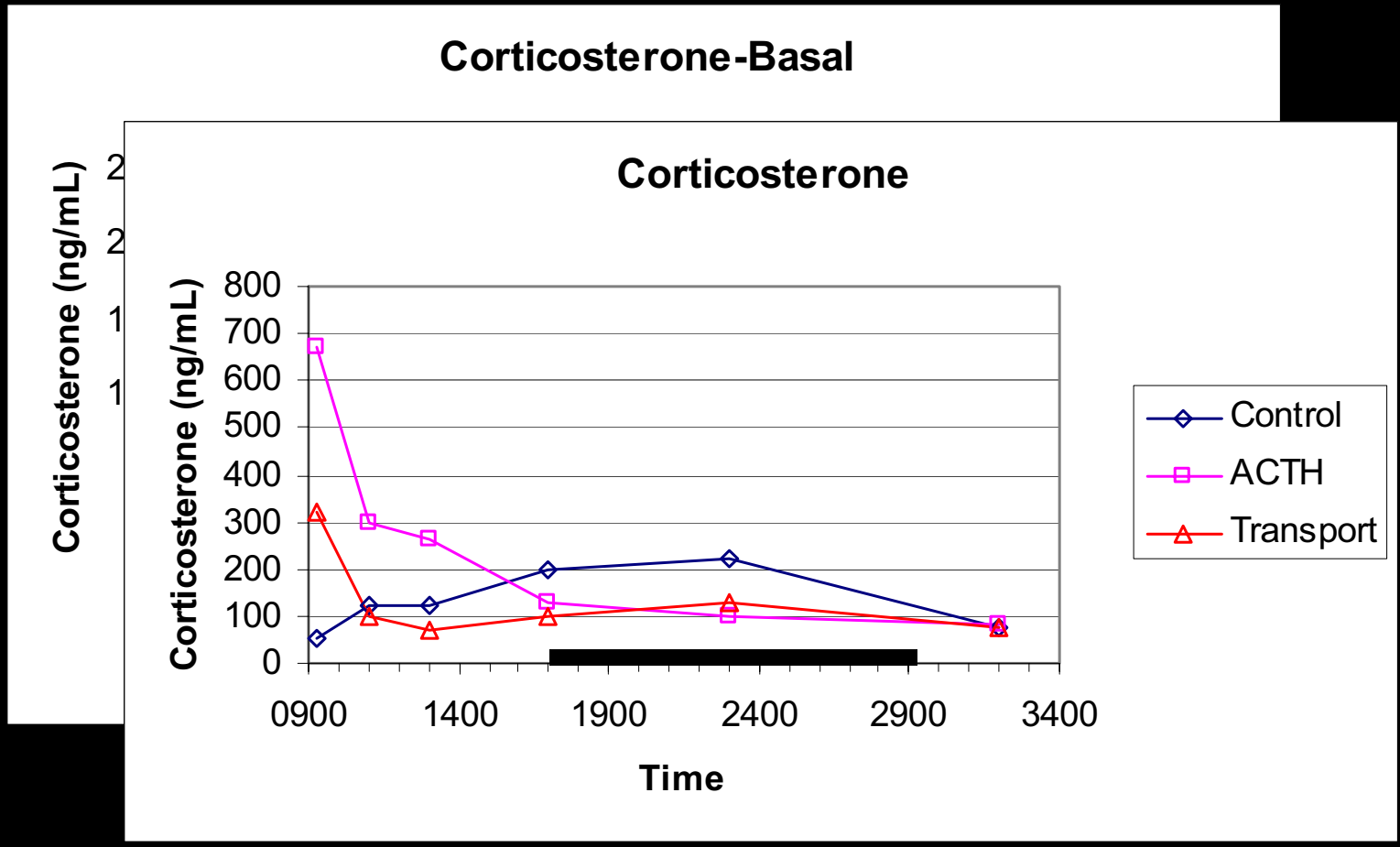
Animal Transport: Effect of Cage Movement

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

Hrs after Transport	Control	ACTH	Transport
0.25	33.7	33.2	33.6
12	33.0	***28.4***	***29.0***
24	32.4	***22.7***	***27.7***

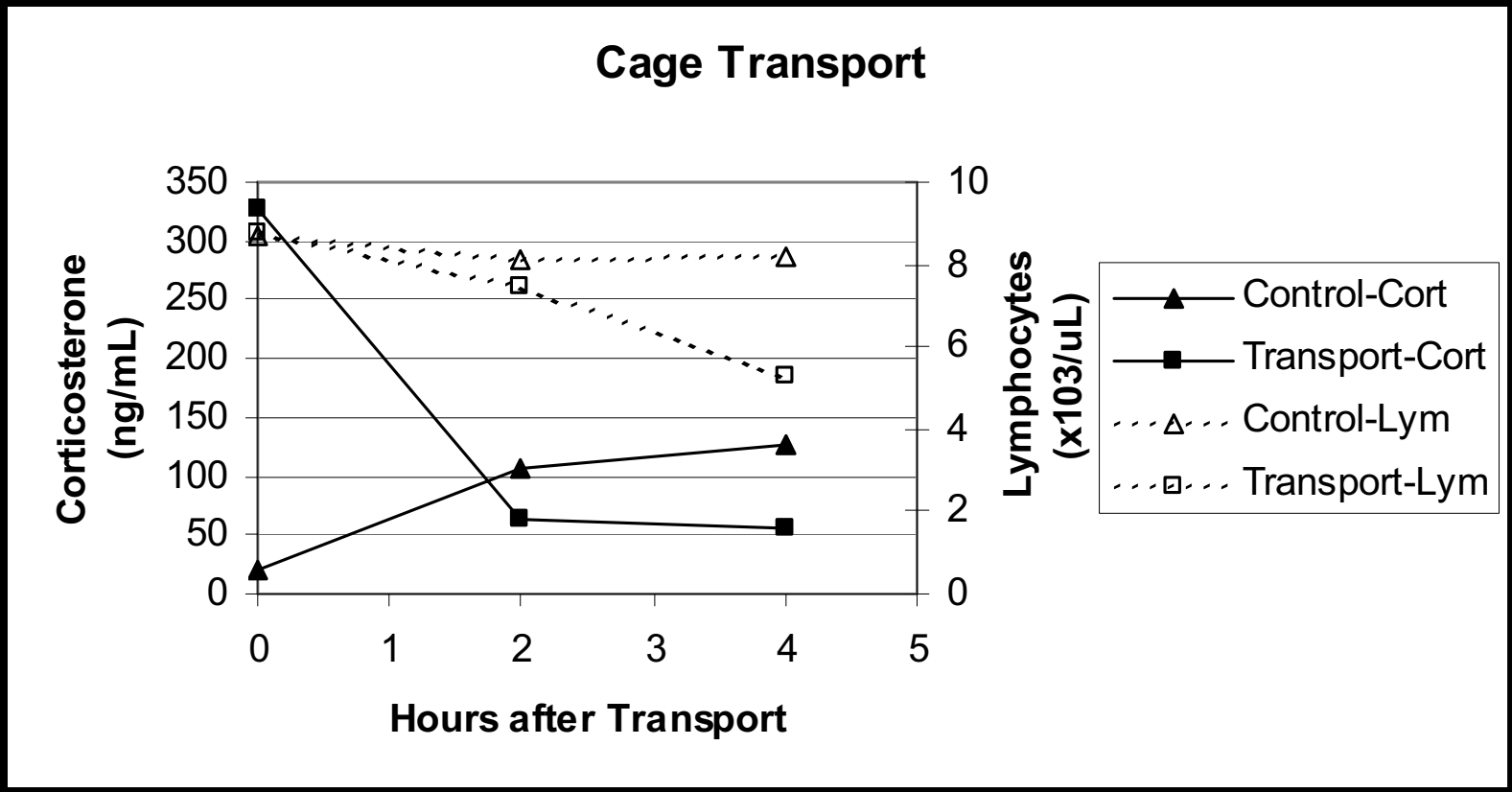
Drozdowicz et al AJVR 51 1841(1990)

Animal Transport: Effect of Cage Movement



Drozdowicz et al AJVR 51 1841(1990)

Animal Transport: Effect of Cage Movement



Drozdowicz et al AJVR 51 1841(1990)

Anesthetics/Blood Collection in Rats

- Anesthetics
  - Isoflurane minimally stressful
  - Generally CO_2 and pentobarbital stressful
- Stress of blood collection depends on
  - Method of collection
  - Rate of blood draw
  - Previous blood collection

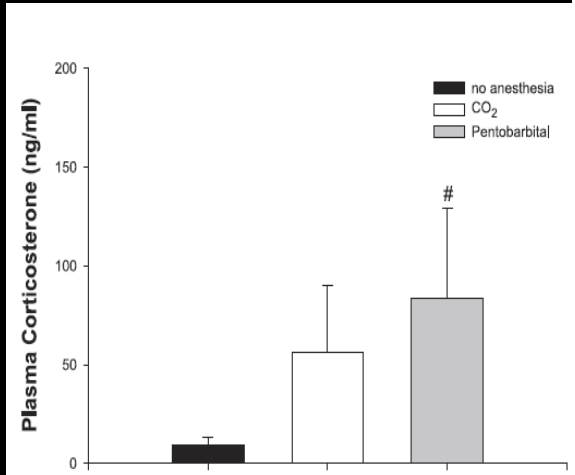


Fig. 4. Plasma ACTH (*top*) and corticosterone (*bottom*) levels in trunk blood from Sprague-Dawley rats undergoing either CO_2 euthanasia, lethal pentobarbital sodium injection, or no anesthesia. $^{**}P < 0.001$, $^{*}P < 0.01$, and $^{\#}P < 0.05$ vs. no anesthesia.

Vahl et al (2005) Am J Physiol Endocrinol Metab 289, E823-8

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)

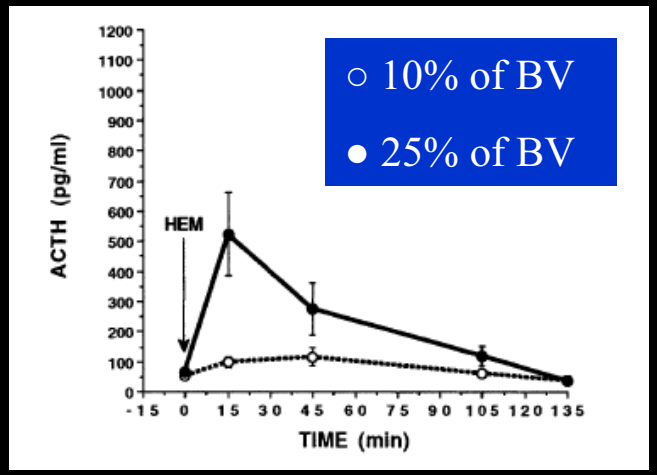
Diehl KH et al (2001) J Appl Toxicol. 21(1):15-23. A good practice guide to the administration of substances and removal of blood, including routes and volumes.

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, norepinenphrine, ACTH and corticosterone
  - ≥ 20% of blood volume: maximum stress
    - ACTH ~5x after collection of 25% of blood volume



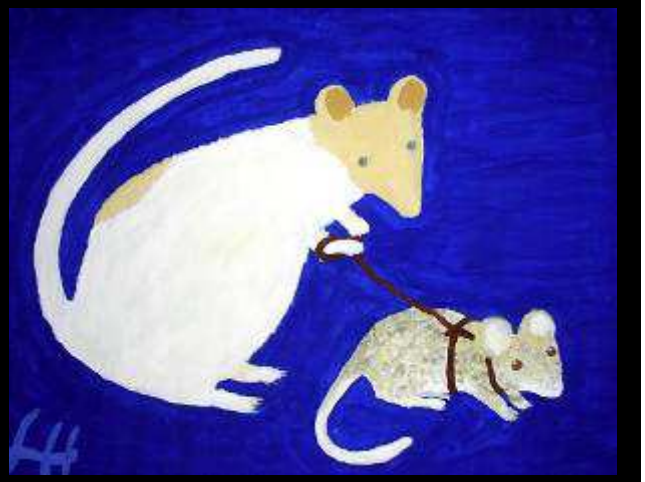
Pacak et al (1998) Am J Physiol R1247-R1255
Graessler et al (1989) Am J Physiol R661-667
Wiersma and Kastelijn (1985) J Endocr 107, 285-92

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



Knol et al (1992) Vet Q 14, 126-9

Knies et al (2005) Inflamm Res 54, Supp1: S32-S33

Effects of Collection Site (mice)

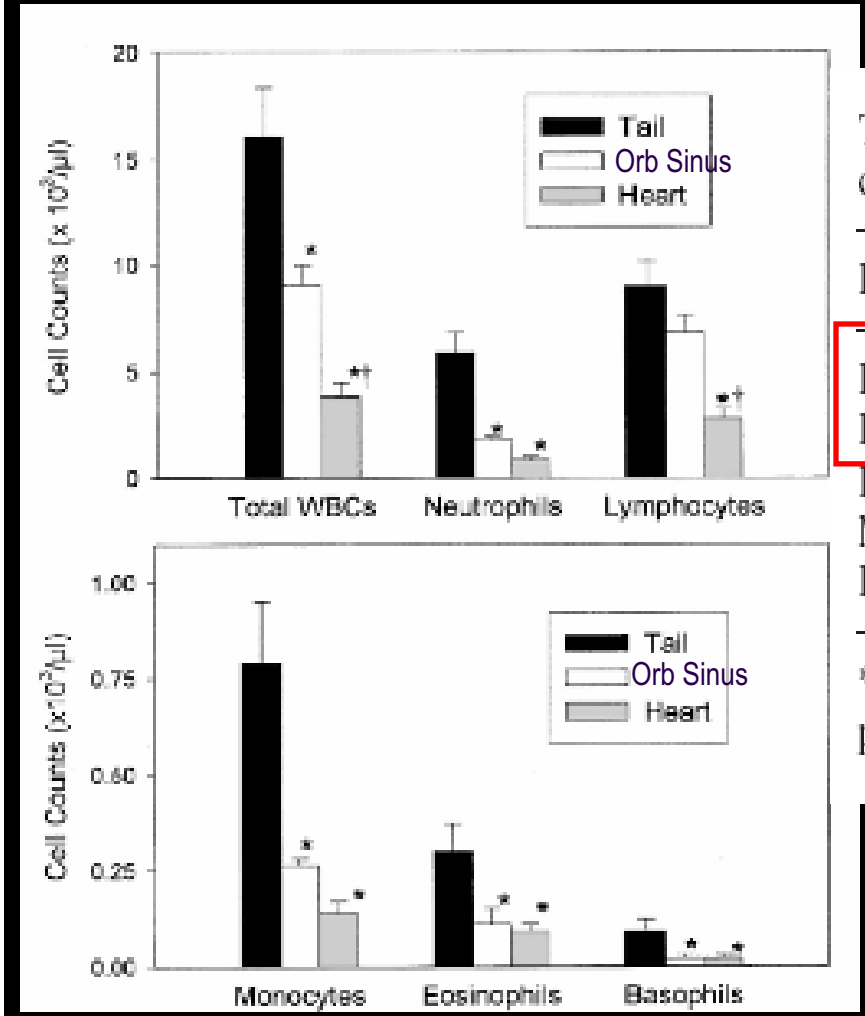


Table 2. Hematological data from blood samples drawn from three different sites.

Parameter	Tail Blood	Eye Blood	Heart Blood
Platelets (×10³/ul)	882 ± 95	637 ± 56	344 ± 73*
Hematocrit (%)	53.15 ± 2.27	46.12 ± 1.90*	41.16 ± 1.11*
RBC (×10⁶/ul)	10.94 ± 0.54	10.14 ± 0.42	9.00 ± 0.22*†
MCV (fL)	48.85 ± 1.94	45.5 ± 0.62	45.5 ± 0.33
Hemoglobin (g/dL)	15.5 ± 1.15	14.28 ± 0.59	13.04 ± 0.34

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

Doeing et al (2003) BMC Clinical Pathology 3:3
Nemzek et al (20010 Inflamm. res. 50: 523–527

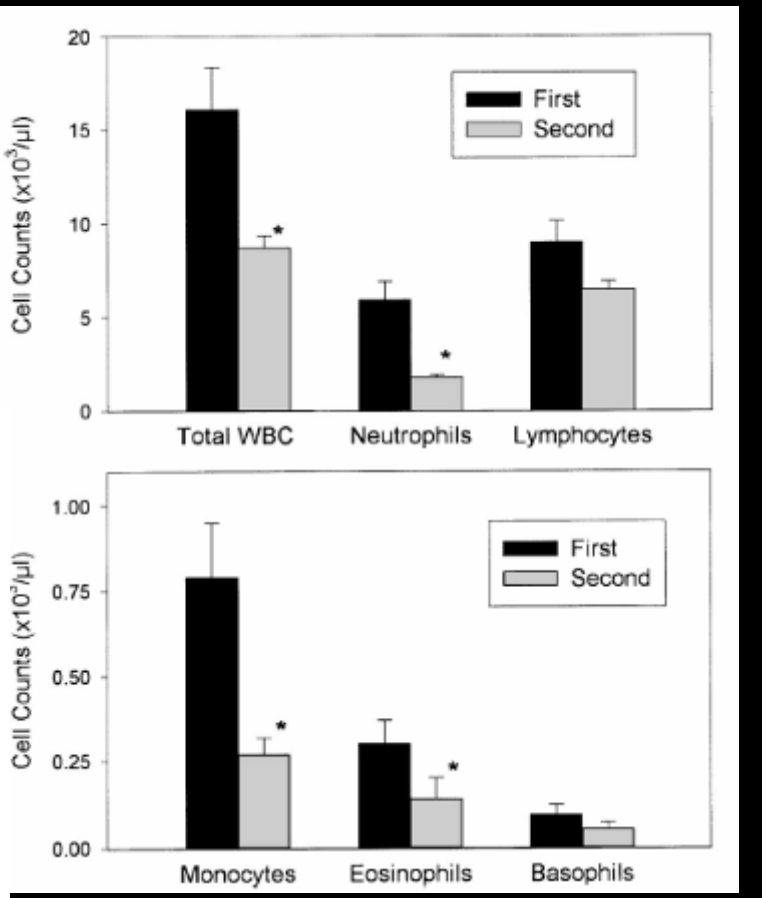
Effects of Previous Collections (mice)

- 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.

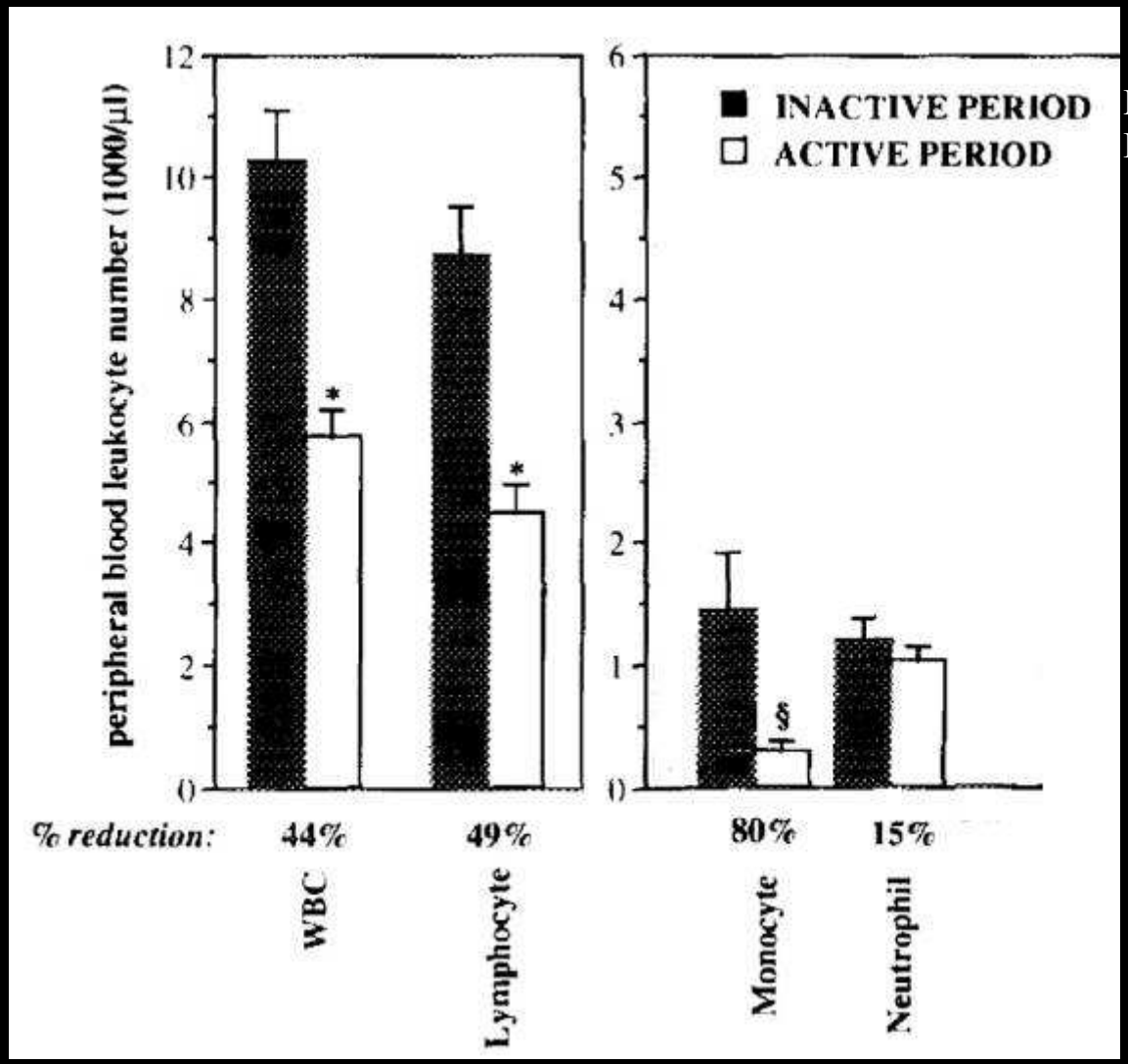
Parameter	First	Second
Platelets ($\times 10^3$/ul)	882 ± 95	563 ± 69*
Hematocrit (%)	53.15 ± 2.27	44.5 ± 2.29*
RBC ($\times 10^6$/ul)	10.94 ± 0.54	9.65 ± 0.05
MCV (fL)	48.85 ± 1.94	46.15 ± 0.43
Hemoglobin (g/dL)	15.5 ± 1.15	12.84 ± 0.59

* = $p < 0.05$.

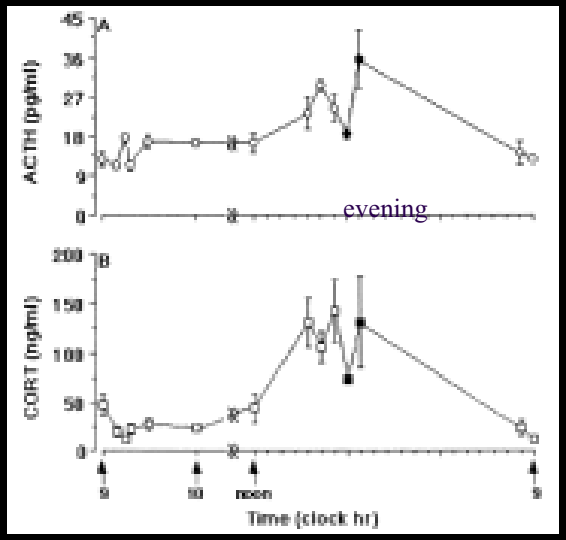


Nemzek et al (20010 Inflamm. res. 50: 523–527

Normal Diurnal Corticosterone Pattern Affects Peripheral Leukocyte Counts



Morning/Day
Evening/Night

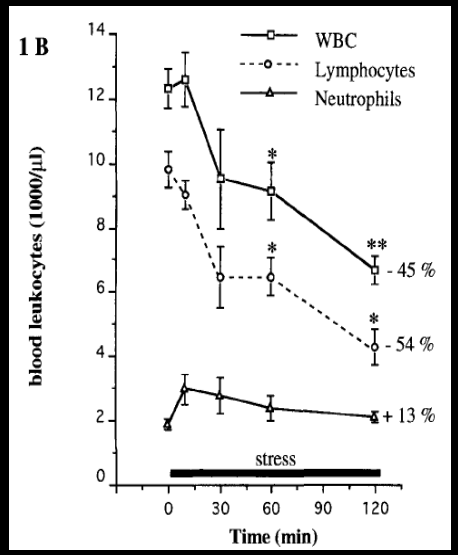


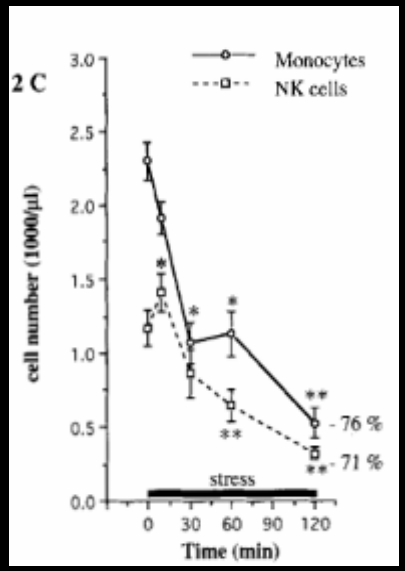
Normal diurnal pattern

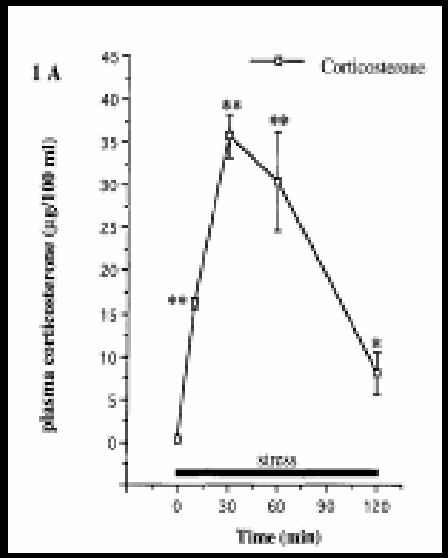
Dhabhar et al (1994) Brain, Behavior, Immunity 8: 66-79
Thrivikraman et al (2002) Brain Res Prot 10, 84-94

Effect of Restraint Stress on Leukocytes (Rats)

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

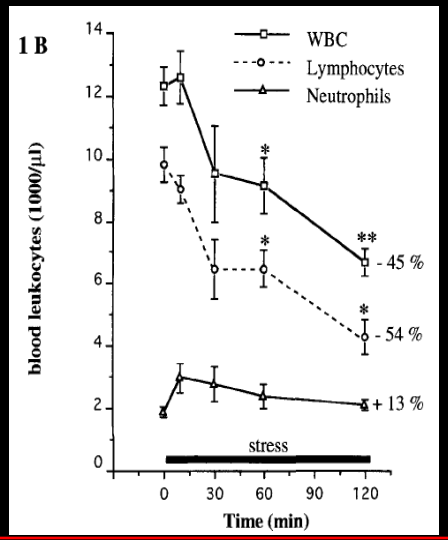


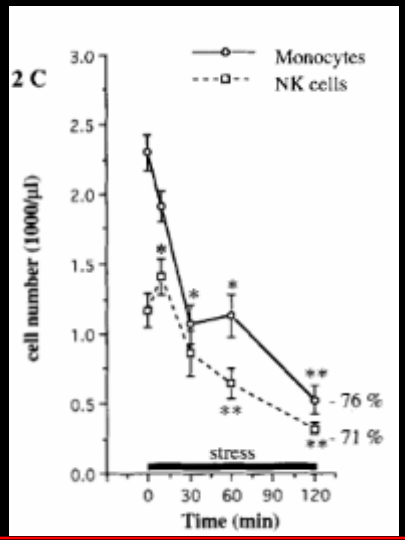


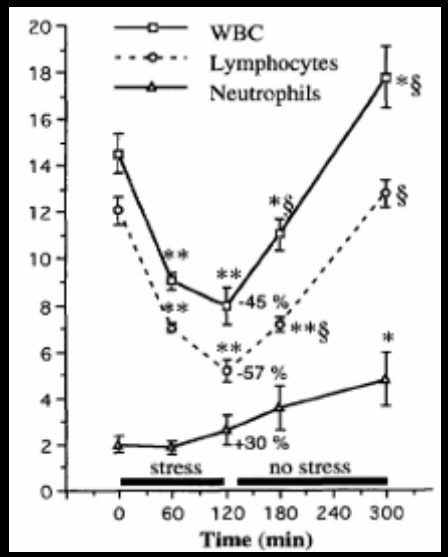


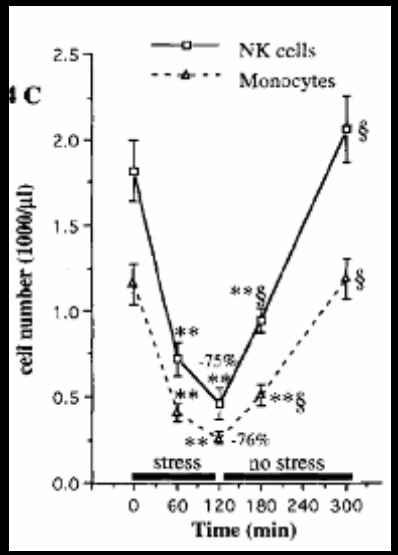
Dhabhar et al (1995) J Immuno 154: 5511-27
Bauer et al (2001) Life Sciences 69: 1167-79
Keller et al (1988) PNAS 85:9297-9301

Rapid Leukocyte Recovery after Restraint Stress









Dhabhar et al (1995) J Immuno 154: 5511-27

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

Szygula et al (1985) Adv Exp Med Biol 191: 571-8
Kuhn et al (1991) J Exp Anim Sci 34: 99-104
Pauluhn et al (2004) Toxicol Sci 81: 198-215

Effects of Preanalytical Stressors on Leukocytes

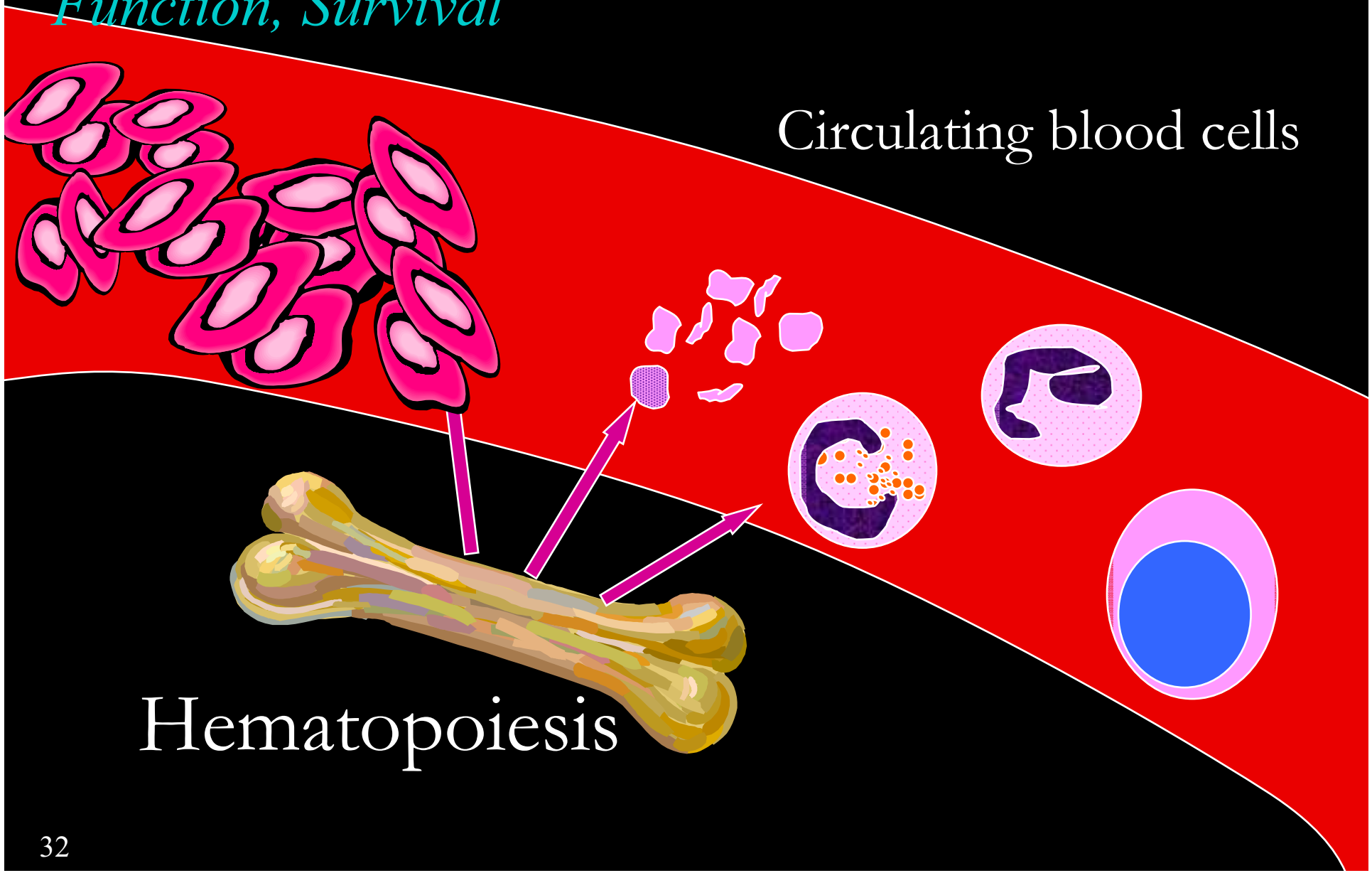
- Epinephrine effects: early (within minutes)
  - ↑ neutrophils (mostly mediated by α-adrenergic receptors)
  - ↑ lymphocytes (mediated by β2-adrenergic receptors)
  - Mostly in proportion to circulating cells
    - Demargination: ↑ blood flow, ↓ adhesion)
    - Contributions from spleen and lungs
-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

Bauer et al (2005) Stress 8: 69-83
Benschop et al (1996) Brain, Behav, Immunity 10:77-91
Schedlowski et al (1996) J. Immunol. 156:93-9

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



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

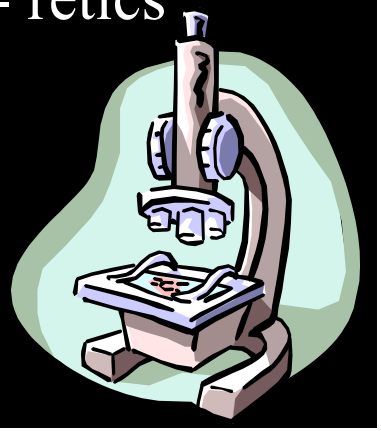
Specific Guidelines for Interpreting Hematology from Rodents vs. Large Animals

- 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

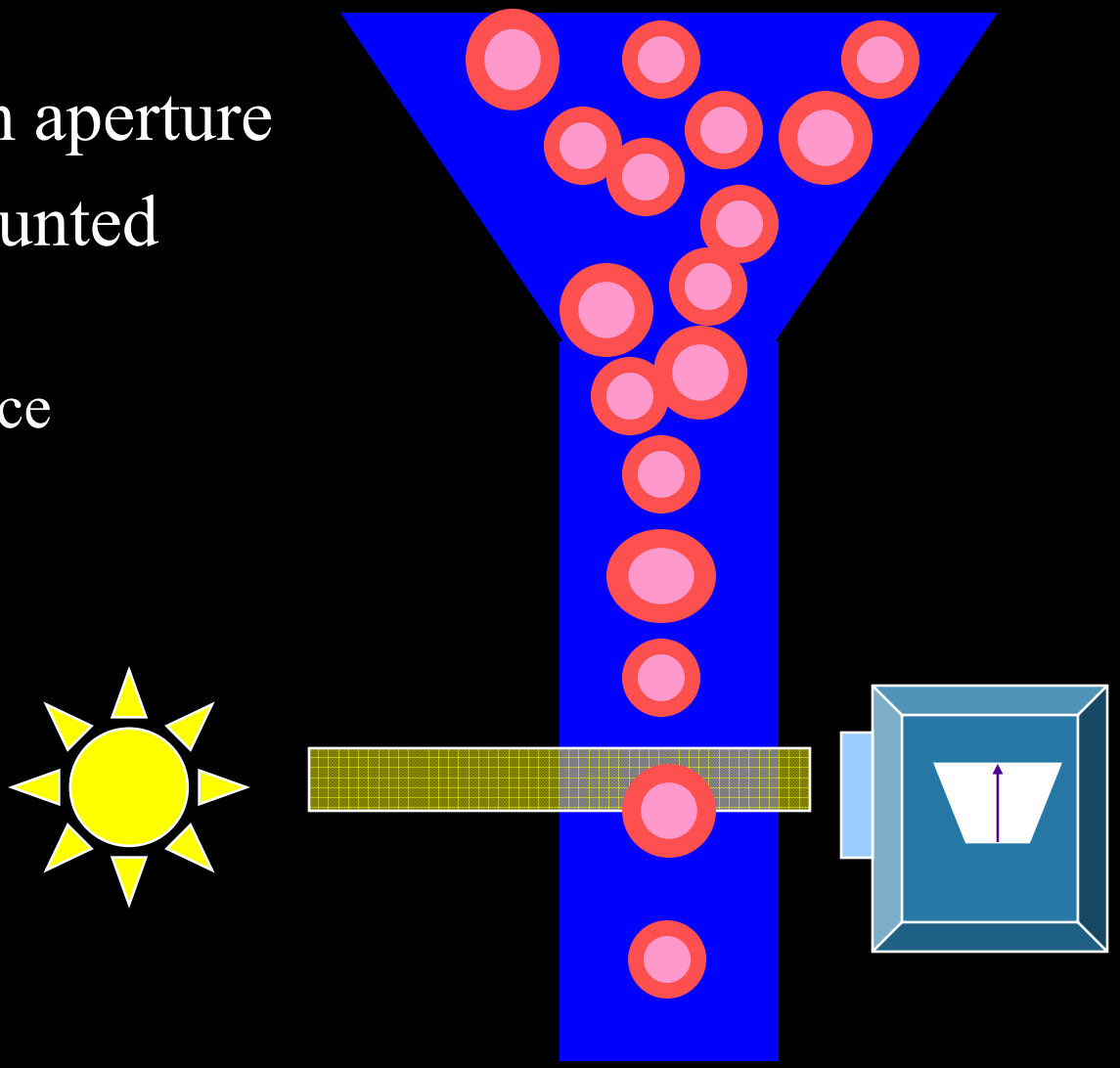
Measured	Calculated
HGB*	HCT
RBC	MCH
MCV	MCHC
RETIC	RDW

*Hemoglobin is measured after RBCs are lysed:

$$\text{Hemoglobin} \xrightarrow{K_3Fe(CN)_6} \text{MetHb} \xrightarrow{KCN} \text{Cyanmethemoglobin}$$

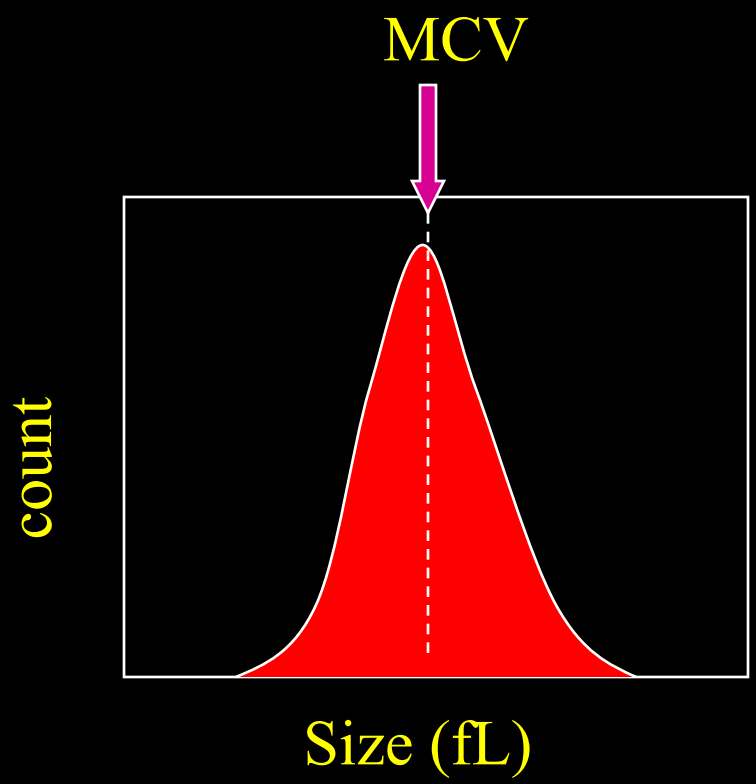
Red Cell Counting/Sizing

- RBCs flow through aperture
- RBCs sized and counted
  - Optical
  - Electrical impedance



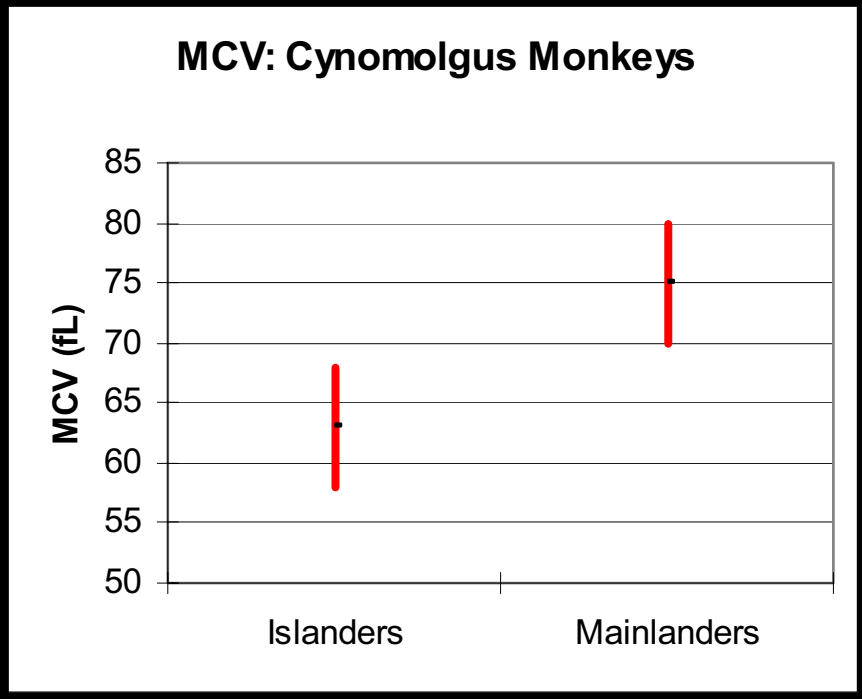
Red Cell Counting/Sizing

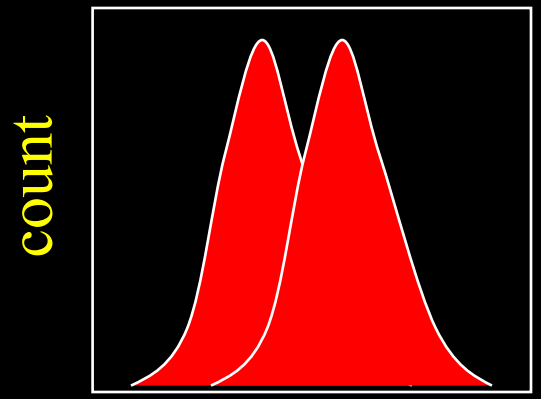
- Histogram created
- Midpoint = MCV
- Count = RBC



MCV and Monkey Source (Cynomolgus)

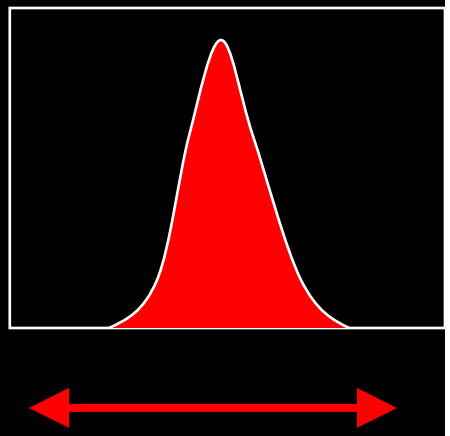
- Mainland Monkeys (China)
  - 70-80 fL
- Mauritius Monkeys
  - 58-68 fL
- Use one source if possible
- Compare to pretest only





Red Cell Counting/Sizing

Measured	Calculated
HGB	HCT*
RBC	MCH
MCV	MCHC
RETIC	RDW**



* Hematocrit = Mean Cell Volume x Red Cell Count

**RDW is a measure of ANISOCYTOSIS

RDW = Coefficient of variation of RBC volume distribution

RDW = [Std Dev x 100]/mean MCV

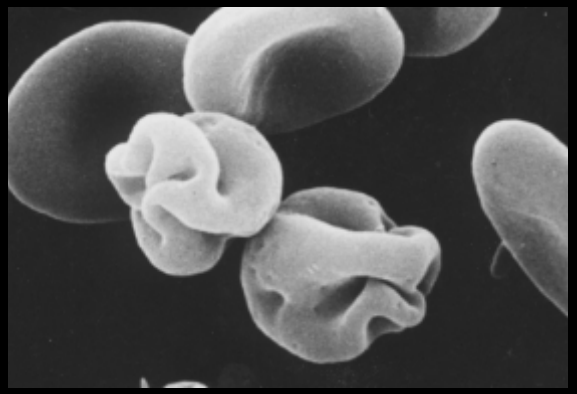
Calculated Red Cell Indices

Measured	Calculated
HGB	HCT
RBC	MCH
MCV	MCHC**
RETIC	RDW

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

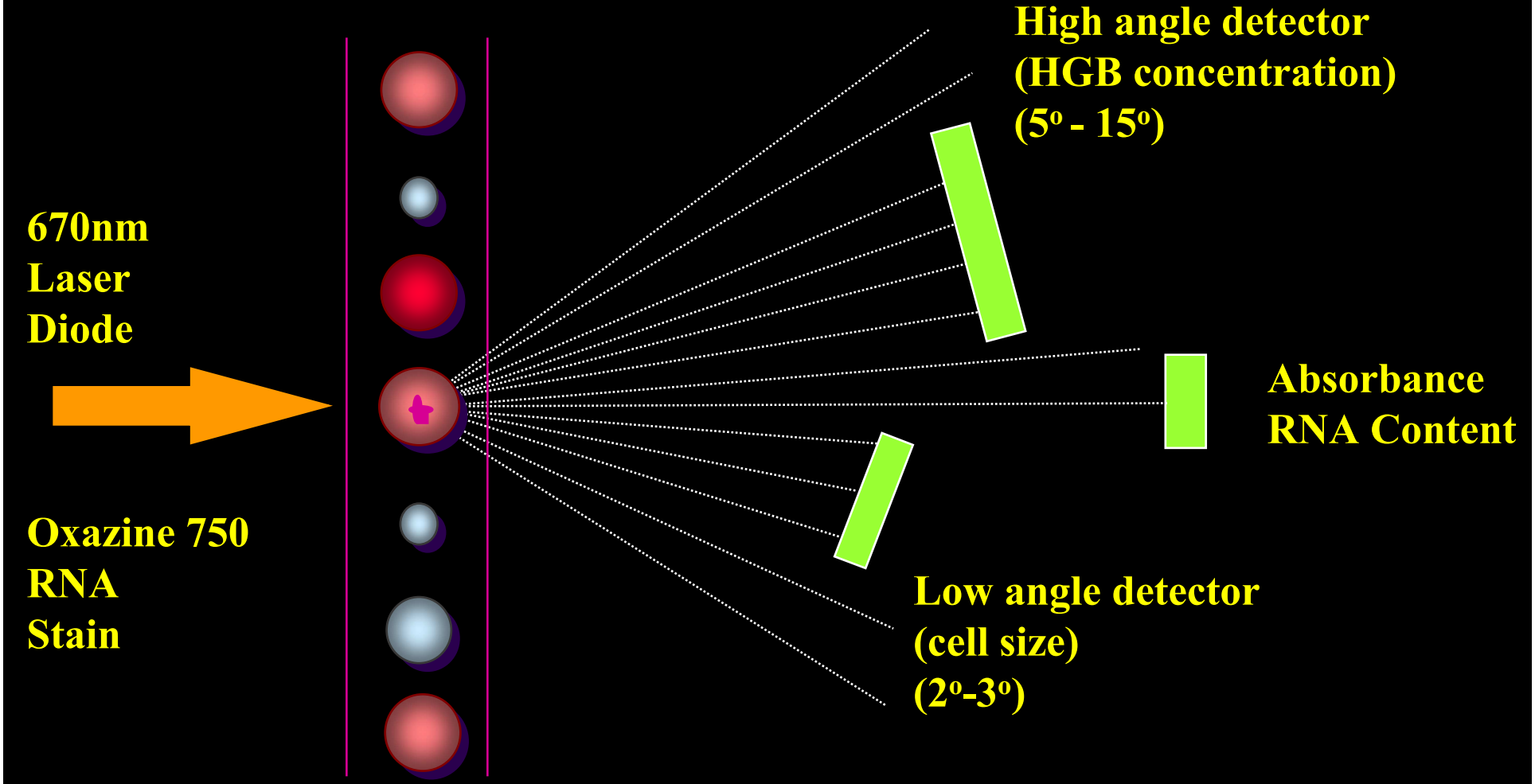
Reticulocyte Counting

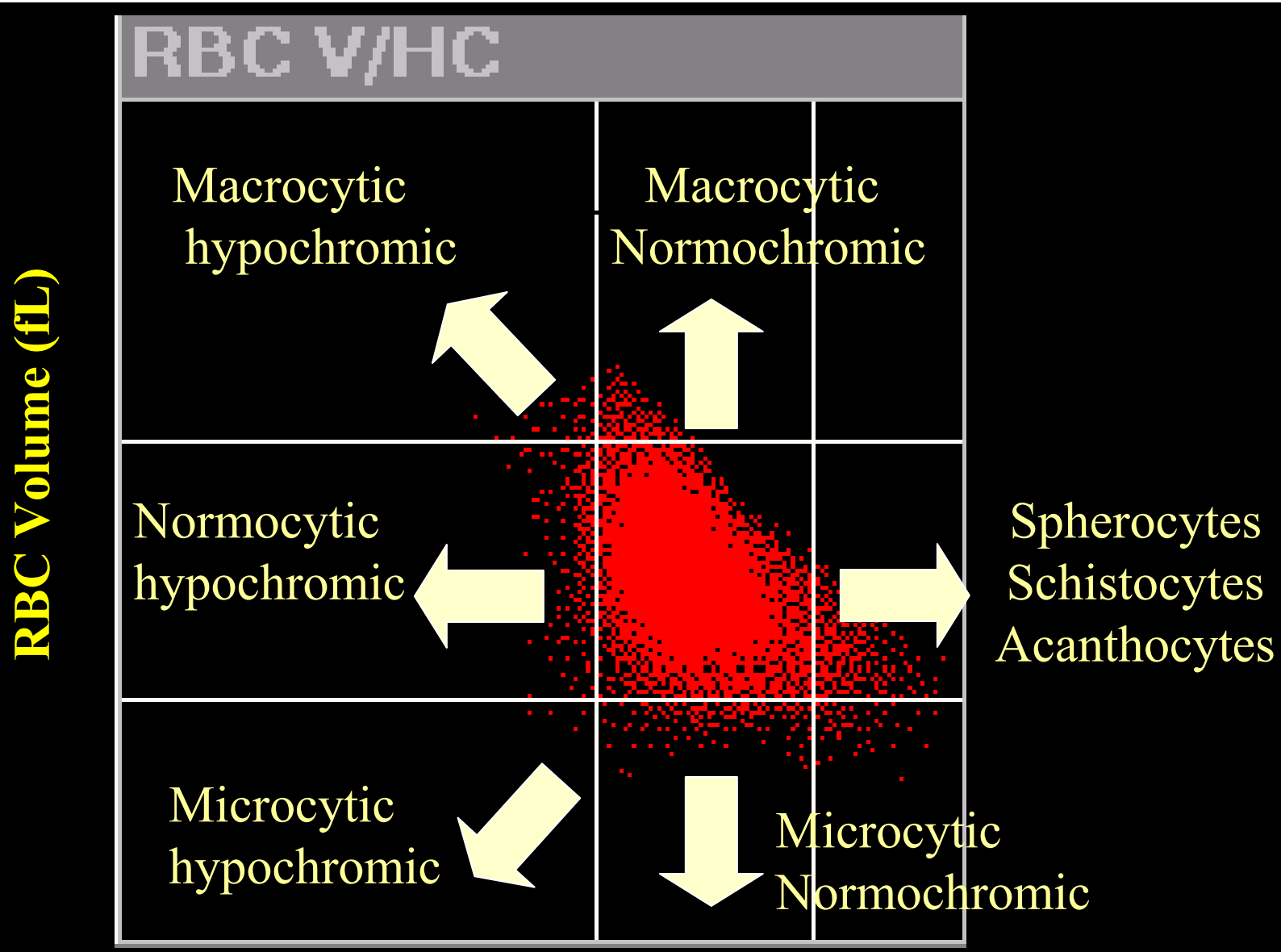
Measured	Calculated
HGB	HCT
RBC	MCH
MCV	MCHC
RETIC	RDW



Always analyze reticulocytes when possible

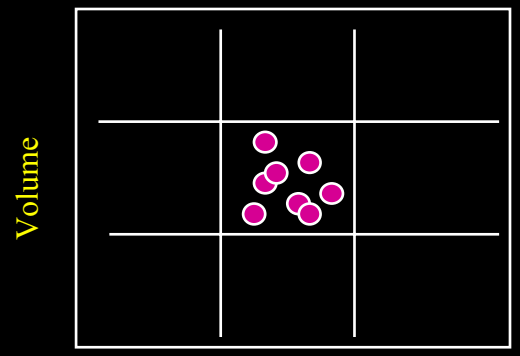
RBC/Reticulocyte Analysis

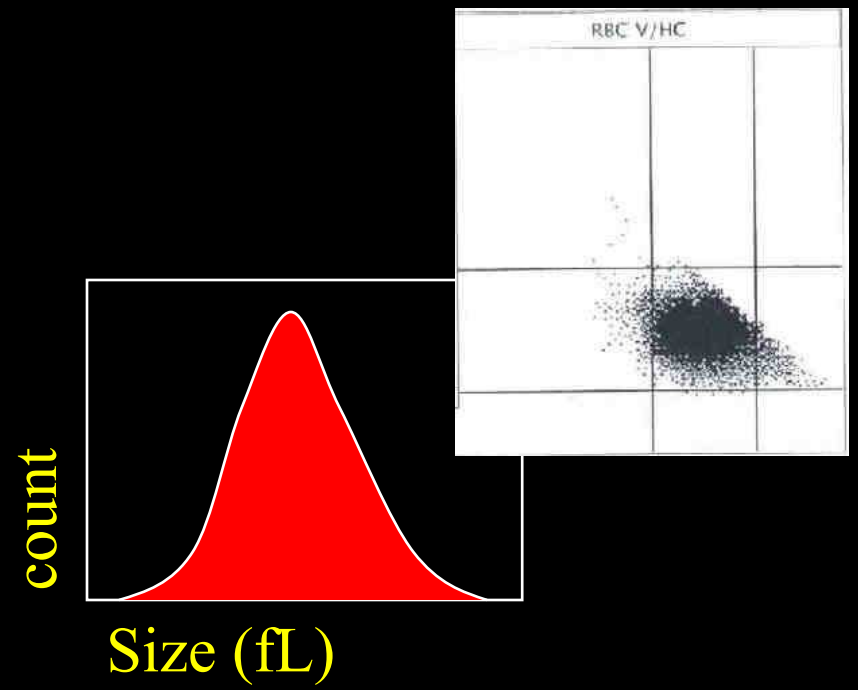




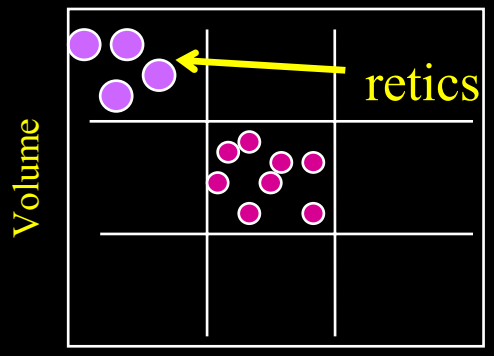
From MHG - Dr. T. Skelton AACC 2001

Normal hemogram

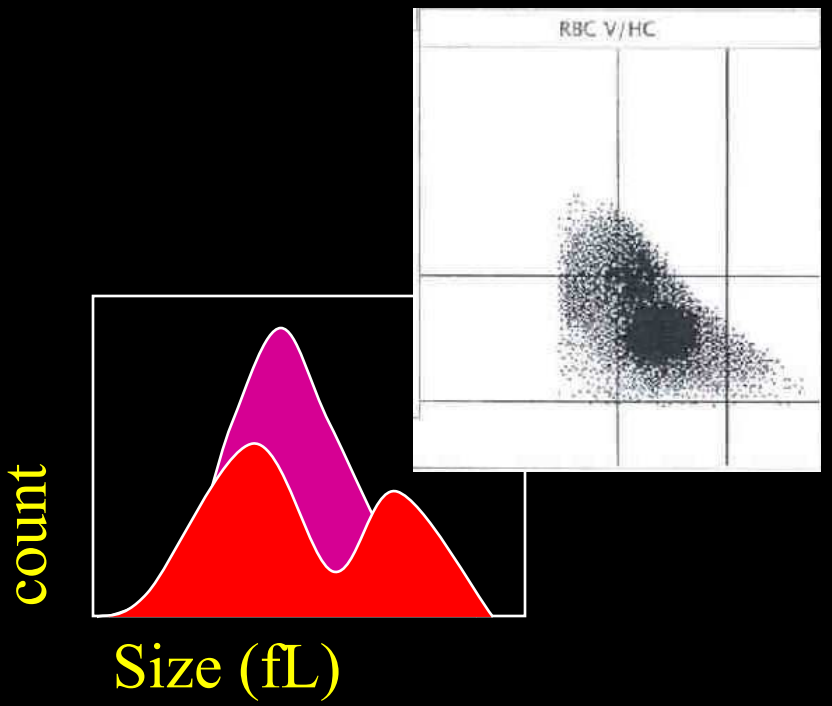




Regeneration



↑ MCV
↓MCHC
↑ RDW
↑RETIC

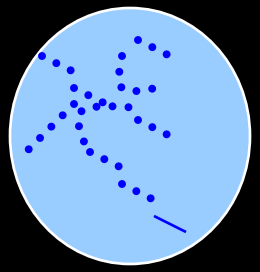


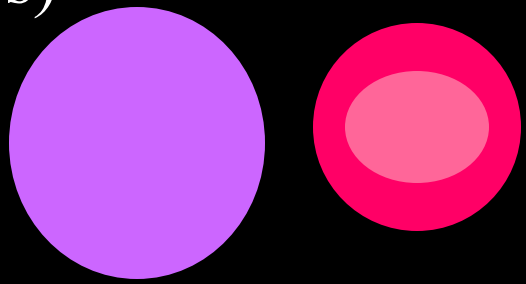
Species Differences: Reticulocytes

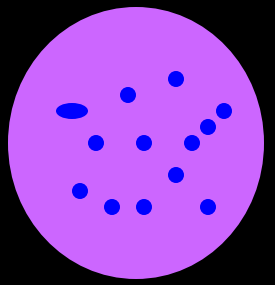
- Rodents vs. larger animals: shorter RBC lifespan, higher retic counts
- RBC lifespan (retic counts)
  - Primates 120 days (27-125 x10^3 cells/µL)
  - Dogs 100-115 days (17-79 x10^3 cells/µL)
  - Rats 45-50 days (135-250 x10^3 cells/µL)
  - Mice 43 days (221-370 x10^3 cells/µL)
- Rodents: more **polychromasia**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

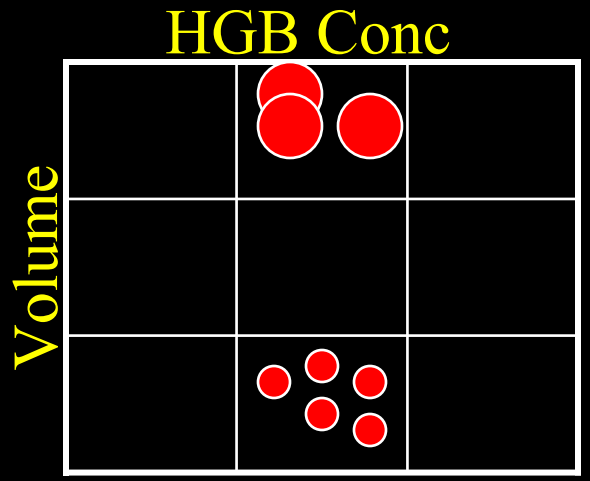
1. Red cell mass parameters
   - Hgb, Hct, and RBC
2. Parameters indicating accelerated erythropoiesis
   - Absolute RET and MCV
3. Other supportive parameters
   - RDW and MCHC
4. Hardly ever useful
   - MCH

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

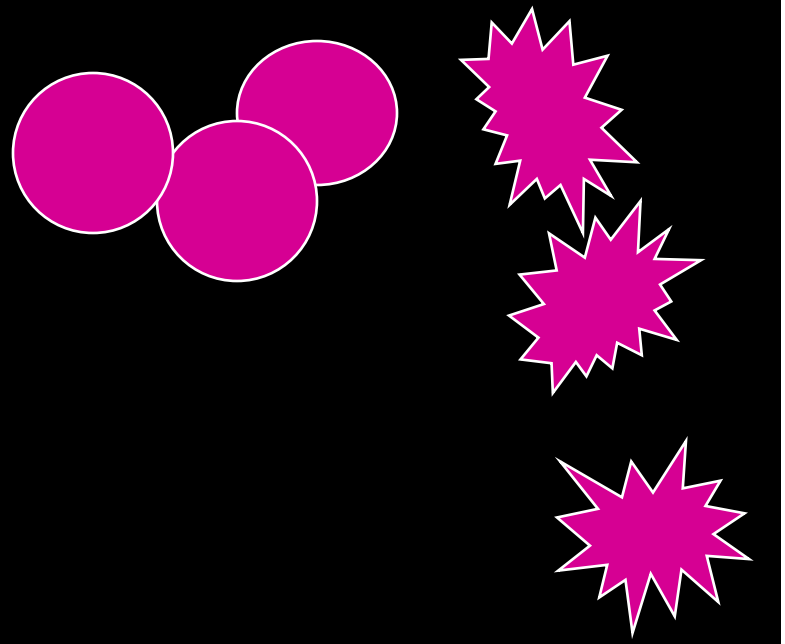
- Most common: due to excessive blood collection
  - Over-collection: effect of drug in anemic animals
  - Collect consistent and reasonable amount of blood from all groups
- Obvious hemorrhage
- Response
  - Release of RBCs by splenic contraction
  - Associated with ↑ neutrophils and platelets
  - Recovery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

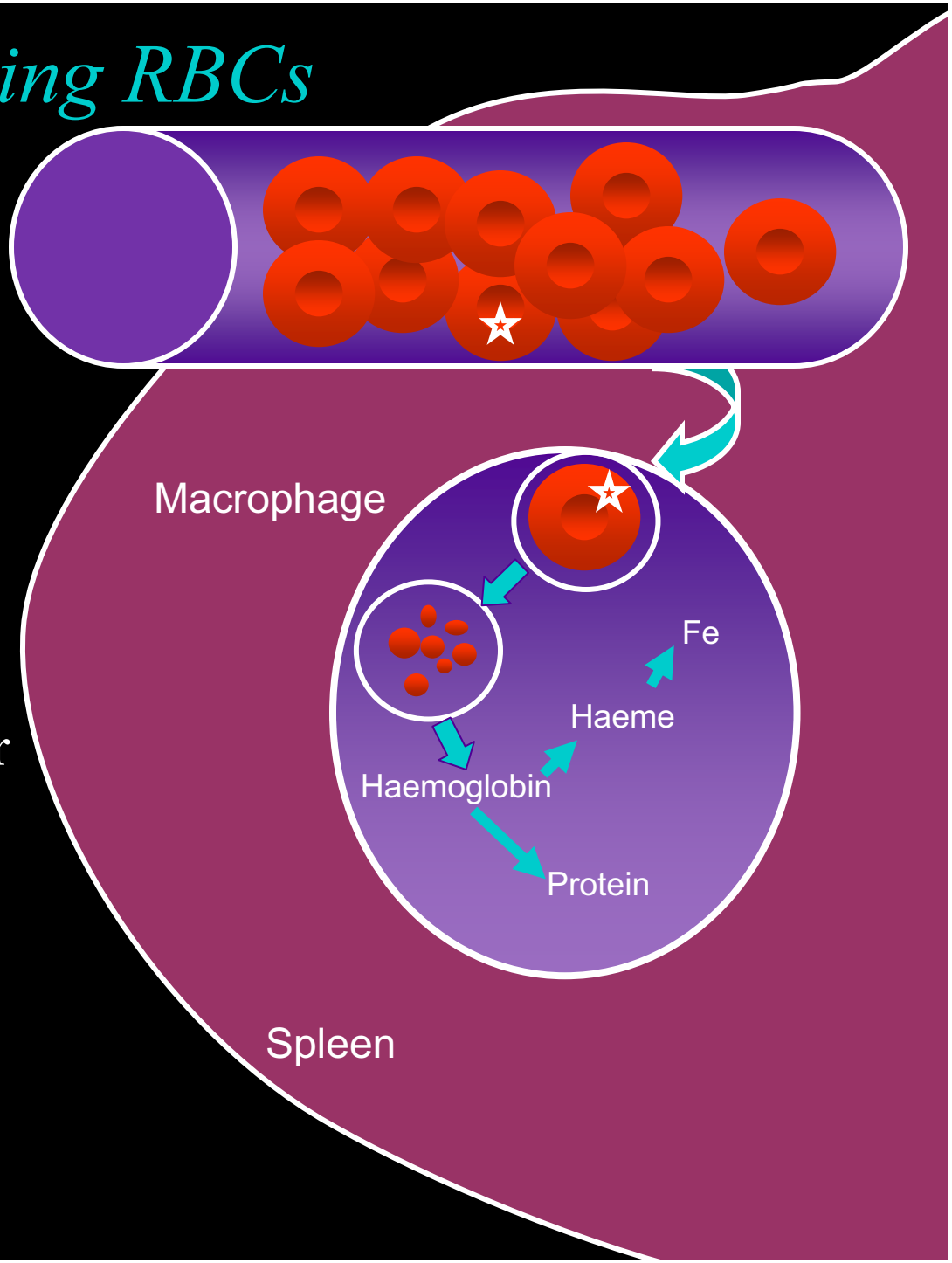
Intravascular

- 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

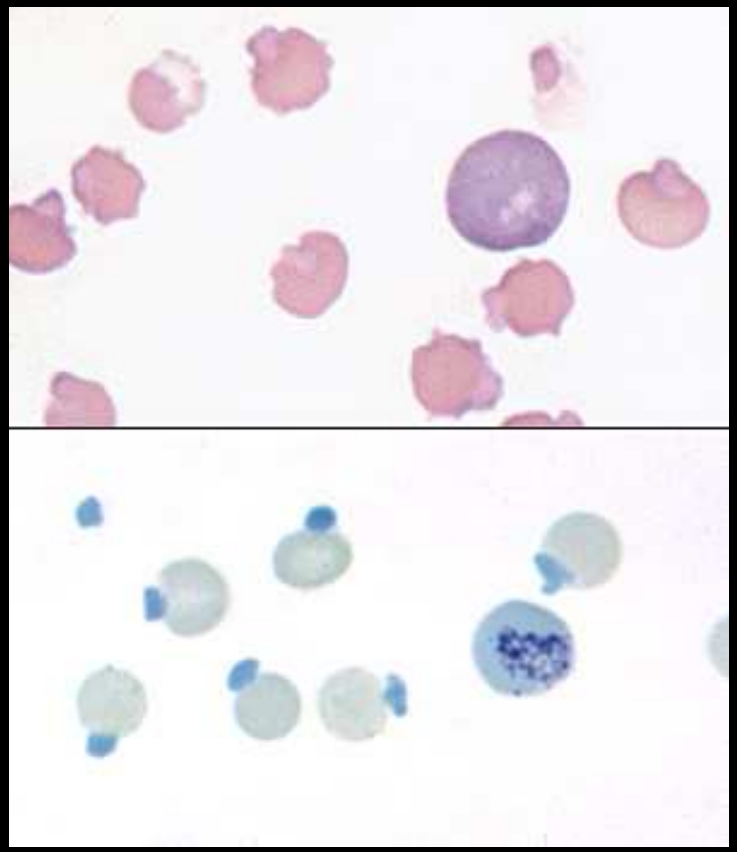
- Two systems prevent oxidative injury
- Methemoglobin reductase system
  - Methemoglobin: oxidized hemoglobin (non-functional)
  - NADH-MetHb reductase reduces to methemoglobin to reduced hemoglobin (functional)
  - Activity in rodents>>dogs, people
- Glucose 6-phosphate dehydrogenase (G6PD) system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

Hemolysis: Oxidation Injury

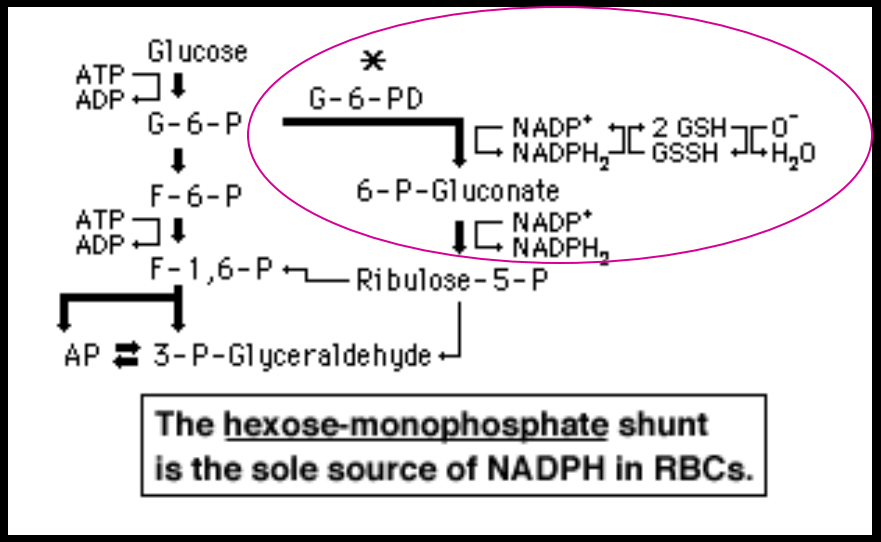
- Evidence of oxidant damage
  - Decreased red cell mass
  - Heinz bodies (denatured hemoglobin)
  - Eccentrocytes
  - Methemoglobin
- If oxidant injury is suspected
  - Prepare new methylene blue stained slides for possible Heinz body enumeration
  - Measure methemoglobin within 30 minutes of blood collection

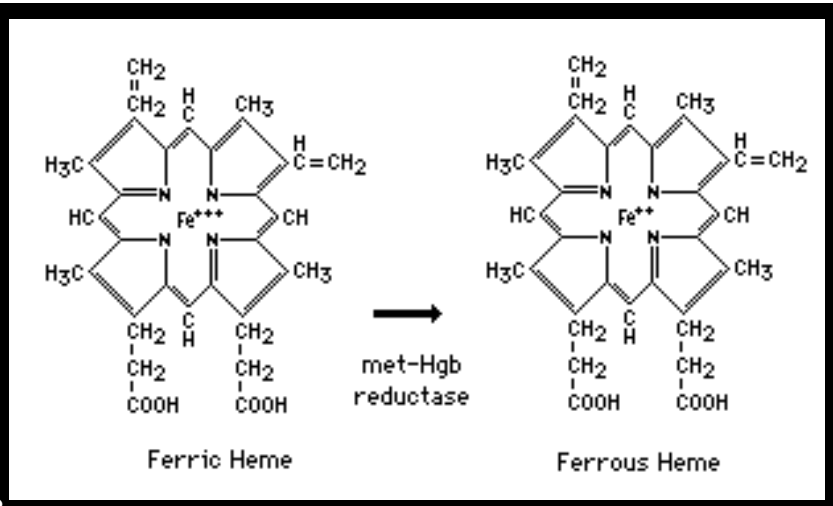
Heinz Bodies (Cat)

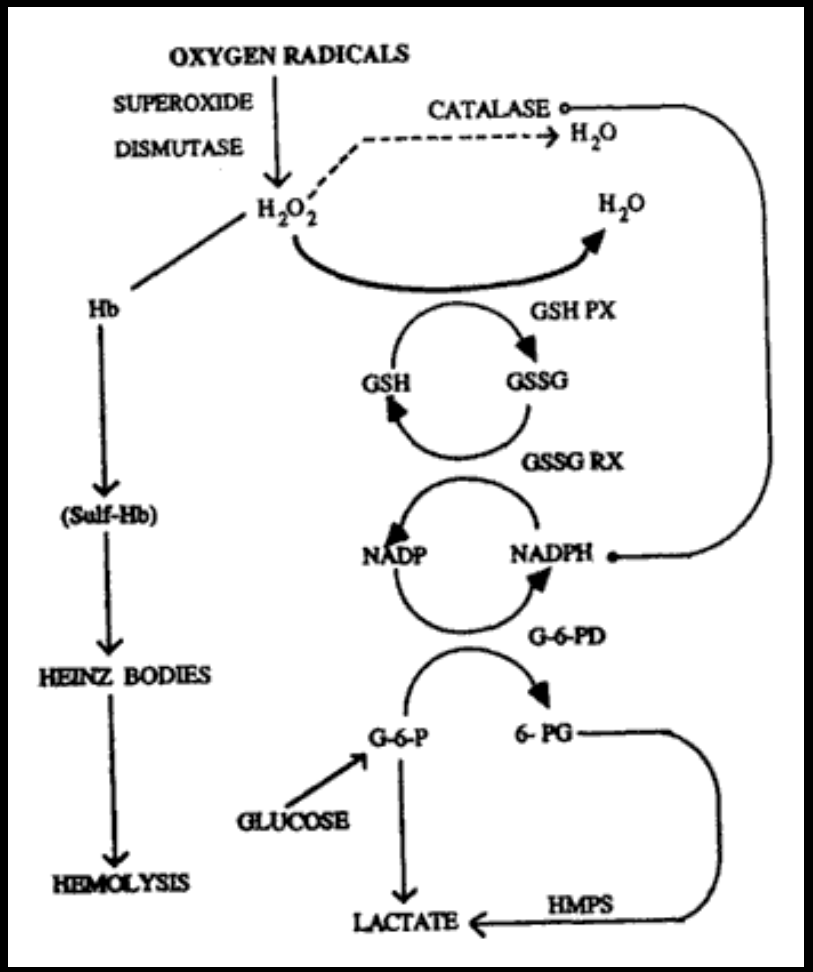
- Wright's-Giemsa Stain
- New methylene blue Stain



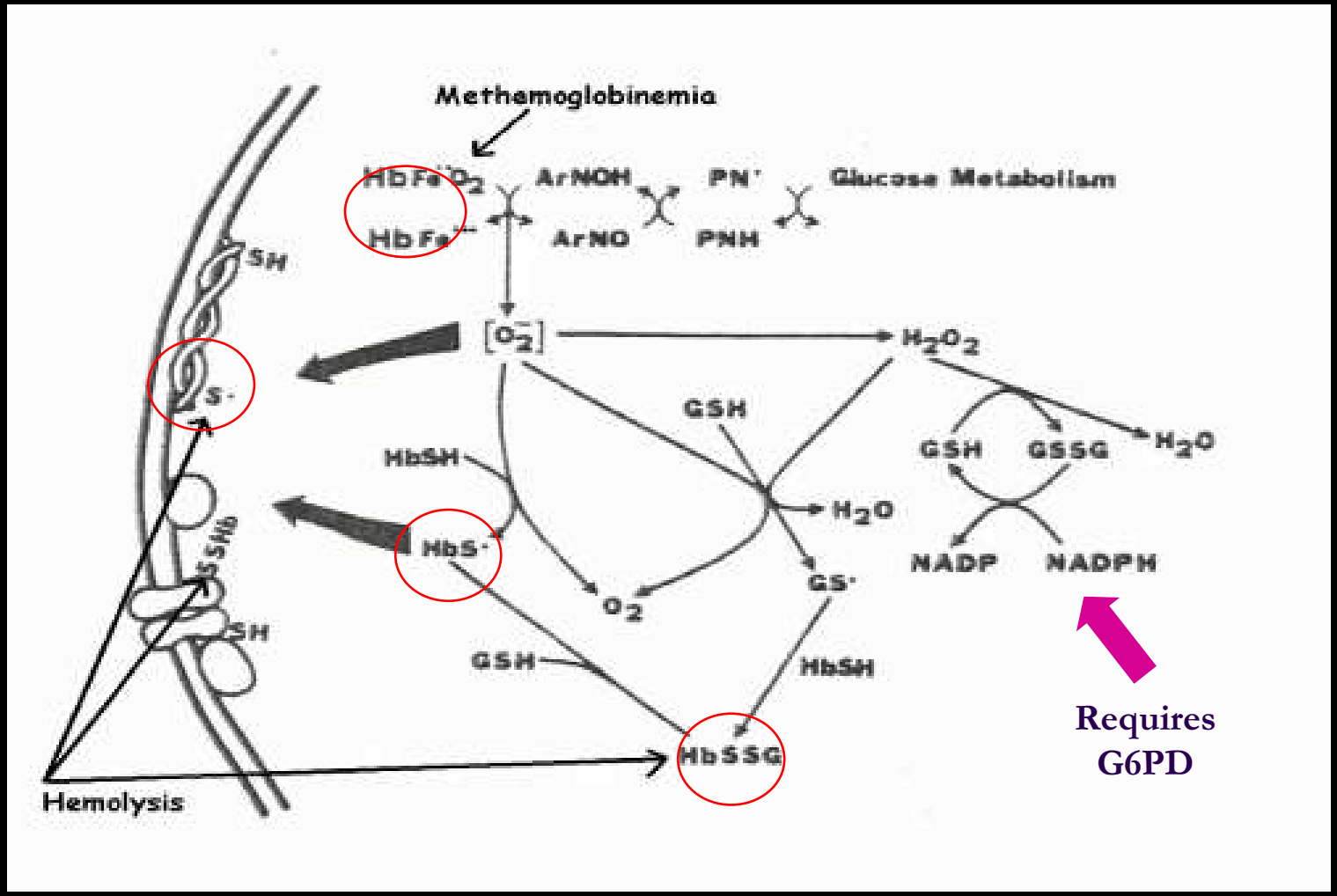
FYI: Hemolysis: Oxidation Injury







FYI: Oxidant Damage to Red Blood Cells

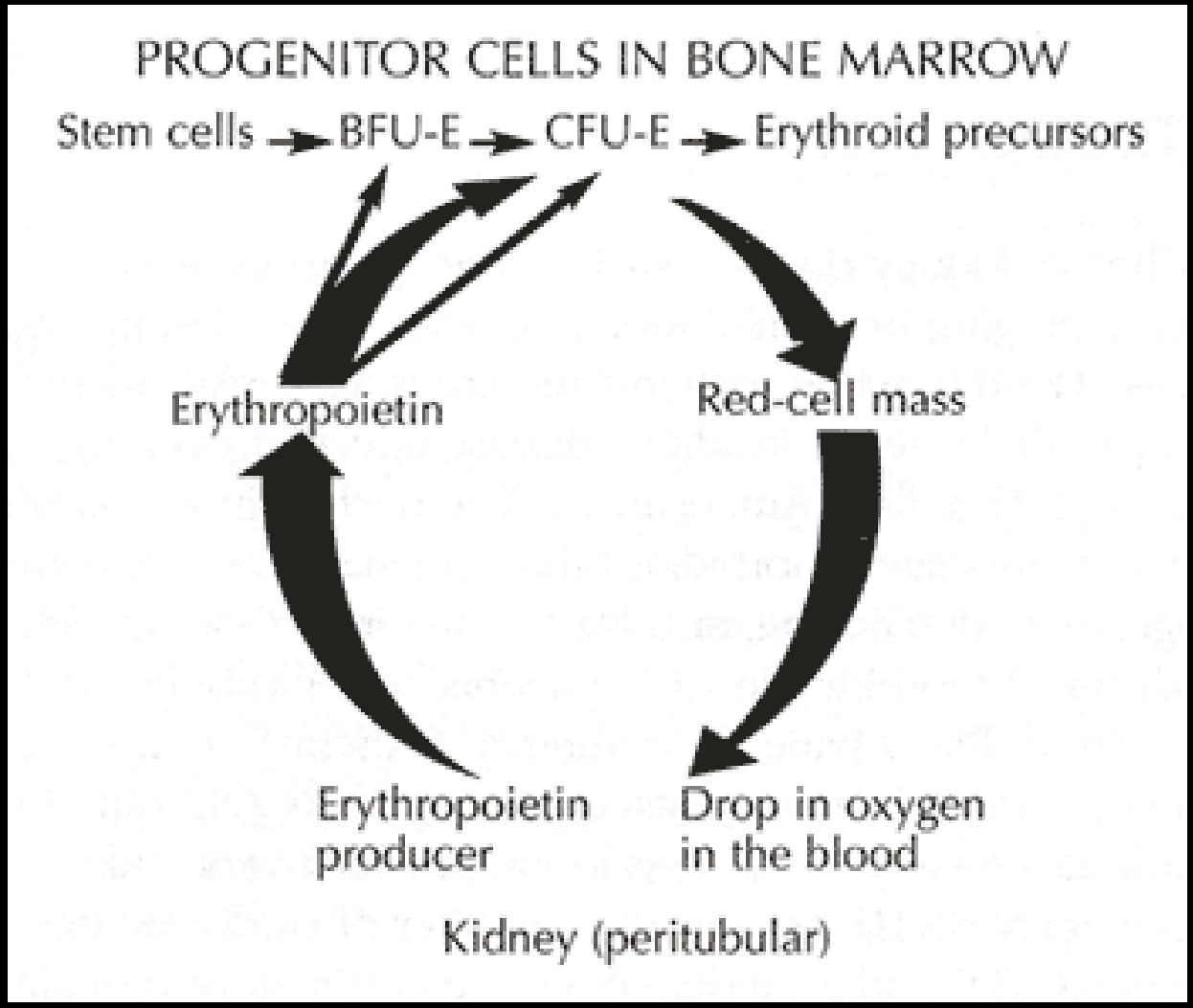


Species Differences: Regenerative Processes

- 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)
- 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



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)
  - 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)
- Peripheral RBC effects causing bone marrow suppression occur **faster** in rodents

Decreased RBC Production

	Regen Anemia (all)	Non-regen dog, NHP	Non-regen rodent
RETIC	↑		
MCV	↑		
RDW	↑		
MCHC	↓		
Polychr	↑		

Decreased RBC Production

	Regen Anemia (all)	Non-regen dog, NHP	Non-regen rodent
RETIC	↑	→	
MCV	↑	→	
RDW	↑	→	
MCHC	↓	→	
Polychr	↑	→	

Decreased RBC Production

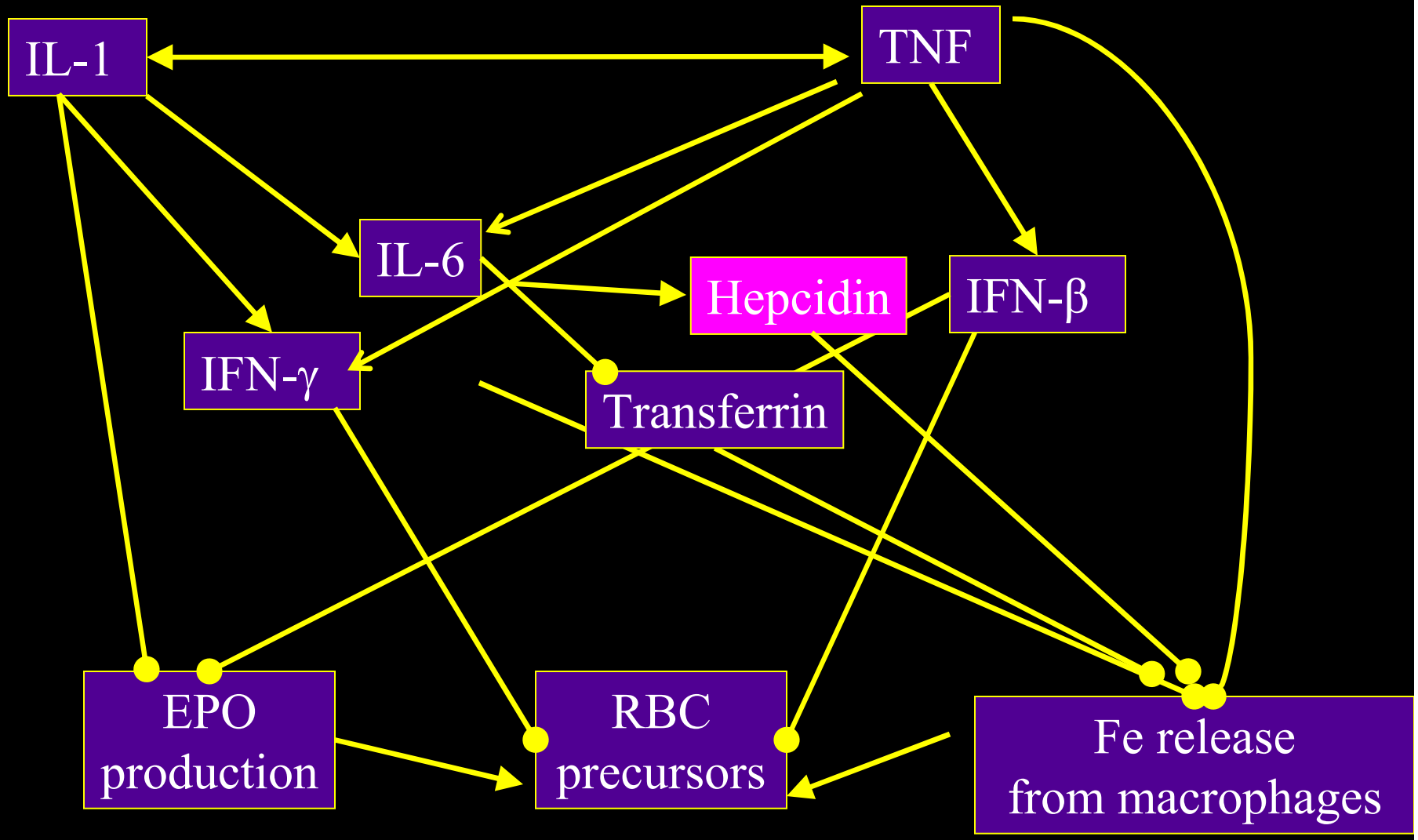
	Regen Anemia (all)	Non-regen dog, NHP	Non-regen rodent
RETIC	↑	→	↓
MCV	↑	→	↓
RDW	↑	→	↓
MCHC	↓	→	↑
Polychr	↑	→	↓

"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)



Ganz et al Hematology Am Soc Hematol Educ Program. 2006;:29-35, 507;
Nairz and Weiss alWien. Klin. Wochenschr. 2006 Aug;118(15-16):442-62.

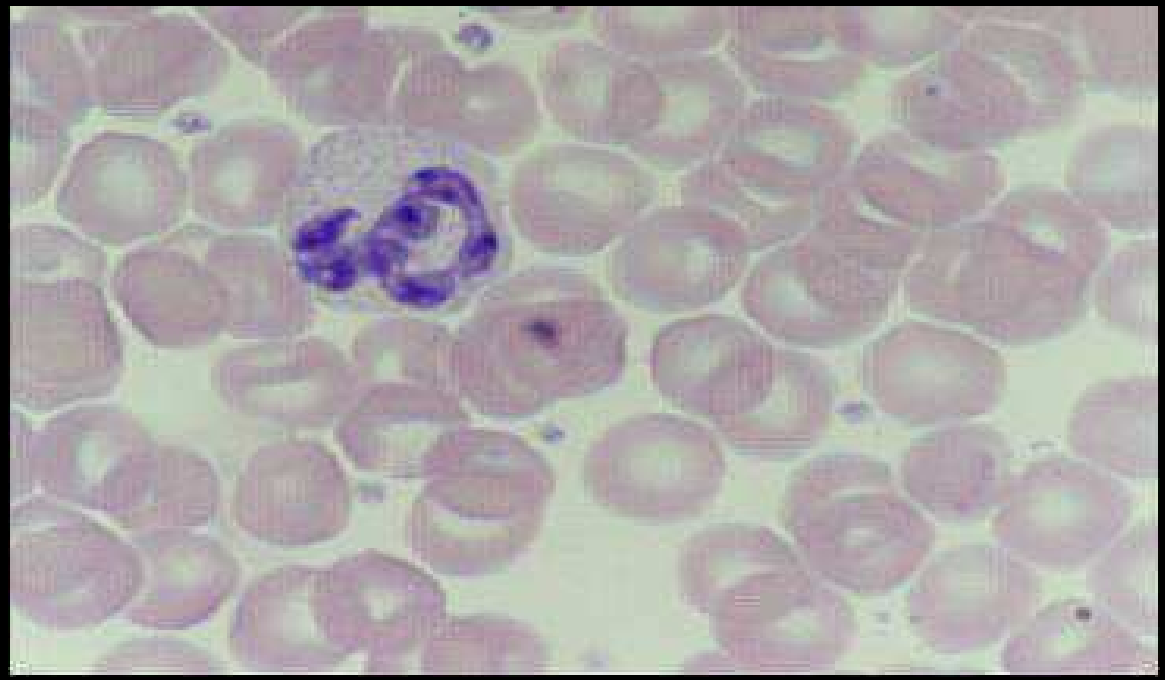
Interpretation of Reticulocyte Responses

- 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

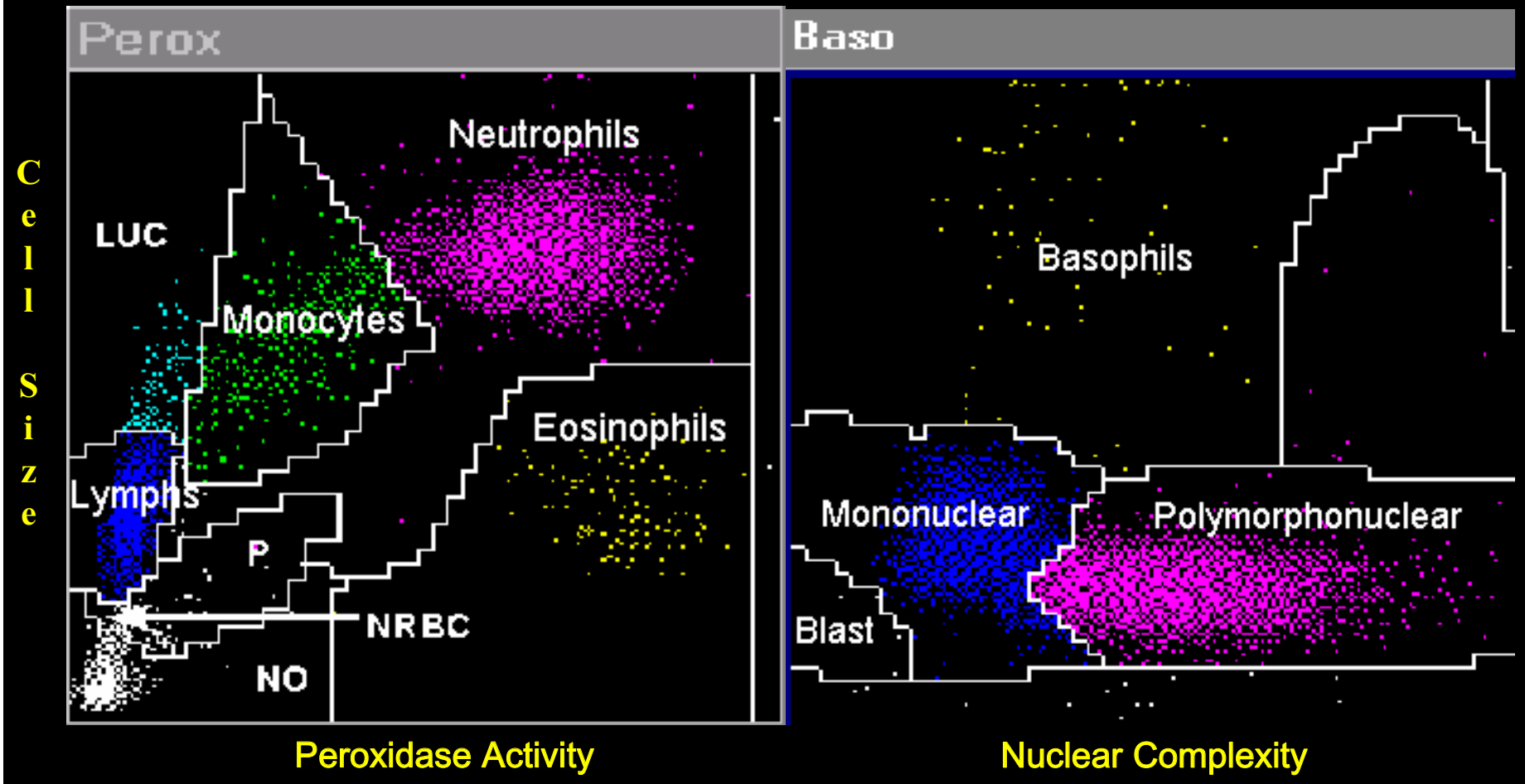
Leukocytes



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

Pool	Neuts	Lymphs	Monos	Eos	Baso
Marrow Storage	Y	N	N	Y	Minimal
Recirculation	N	Y	N	?	N
Marginal and circulating pool	Y	Y	Y	Y	?
Blood transit time	10 hrs	Hours-years	18-23 hrs	Minutes	6 hrs
Tissue half-life	1-2 days	Hours-years	Differ-entiate	Unknown	? up to 2 weeks

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

Bauer et al (2005) Stress 8: 69-83

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.

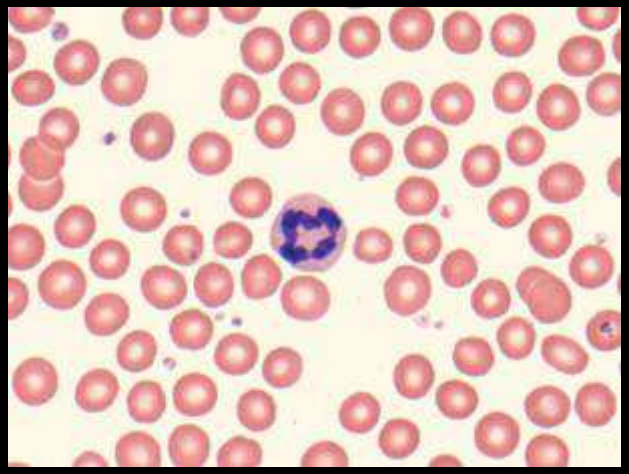
Increased Leukocytes: Inflammation

- 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)

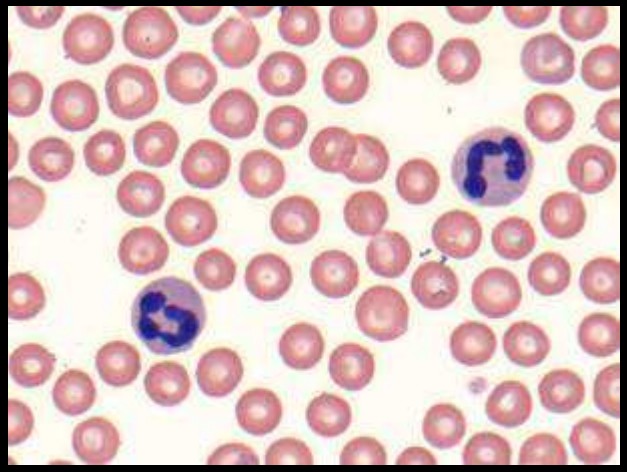
WBC Morphology: Neutrophils

Compared to dogs, rats and mice have…

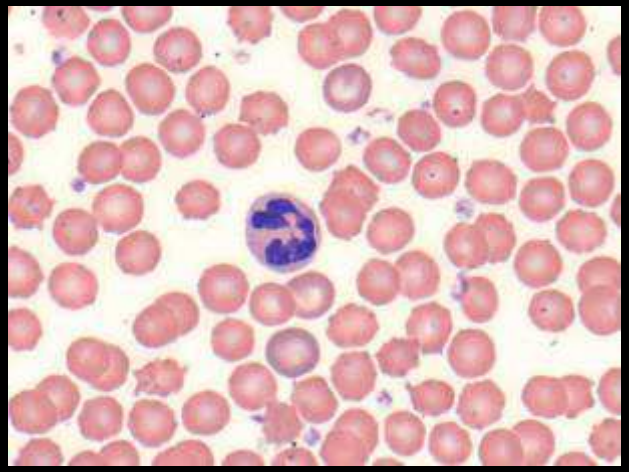
- More neutrophil segmentation
- Fewer bands



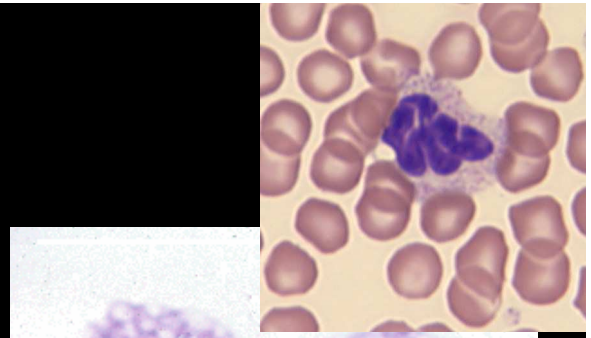
Rat

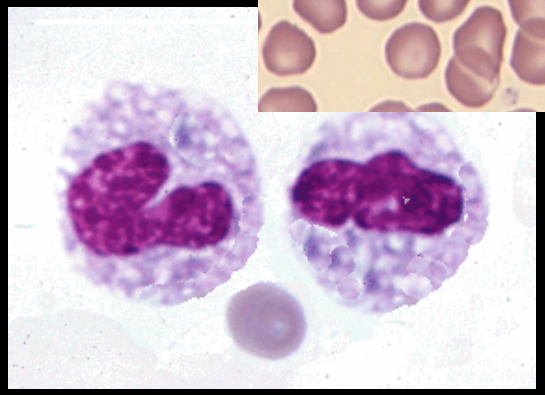


Dog



Neutrophil Morphology during Increased Production

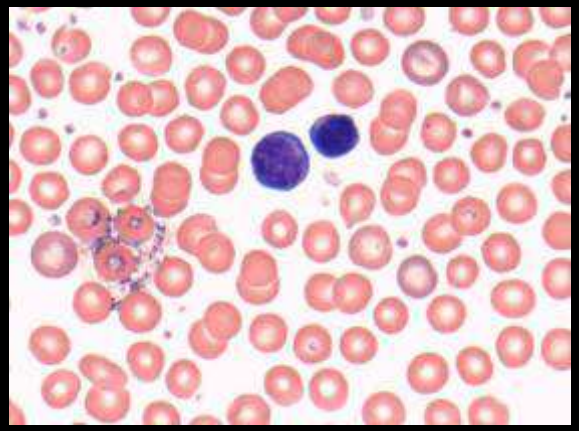


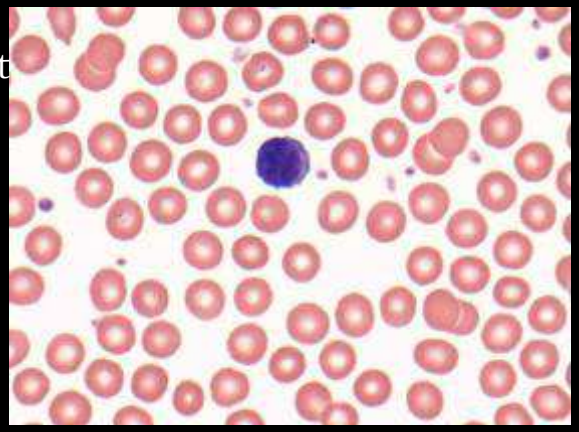


- May see no change, or
- Band neutrophils
- "Toxic" neutrophils
  - Caused by accelerated production
  - Basophilia, vacuolation, Döhle bodies, toxic granulation
- Ring-form nuclei (rodents, other lab animals)
- Comments re neutrophils apply to heterophils
  - Functionally similar to neutrophils
  -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

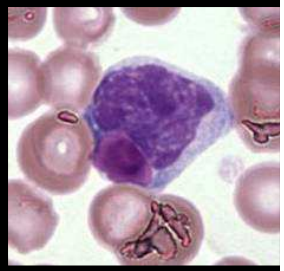
WBC Morphology: Lymphocytes

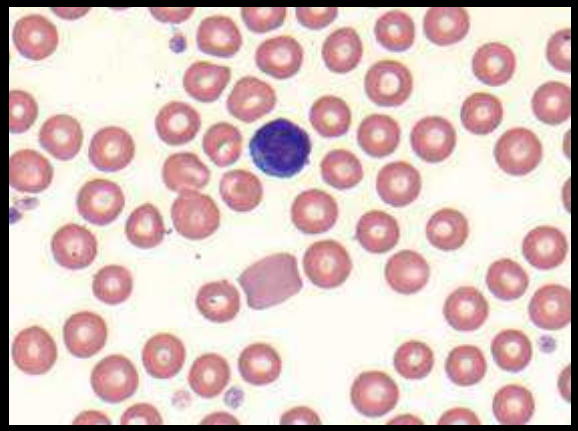
- Mostly small lymphocytes
- Some atypical lymphs
- Kurloff's bodies in some species (guinea pigs)





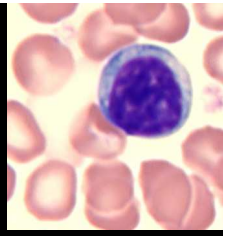
Rat





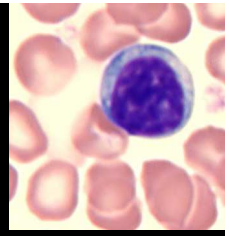
Dog

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

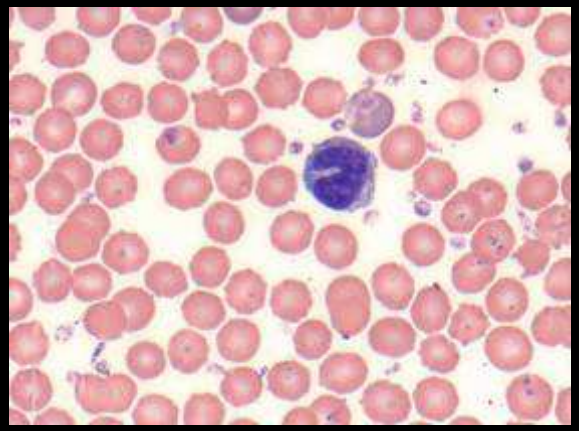
Decreased Lymphocytes

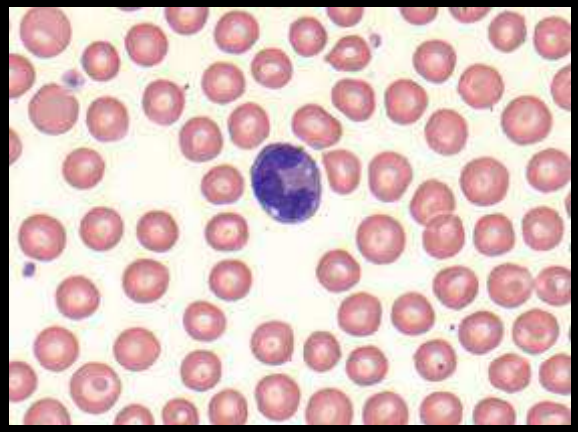


- Drugs directly affecting lymphocytes (other lectures)
- Glucocorticoid-related
  - Redistribution of lymphocytes
  - May not see neutrophil effect in lab animal species with lymphocyte predominance
  - Lympholysis
- Interference with lymph circulation
- Infection

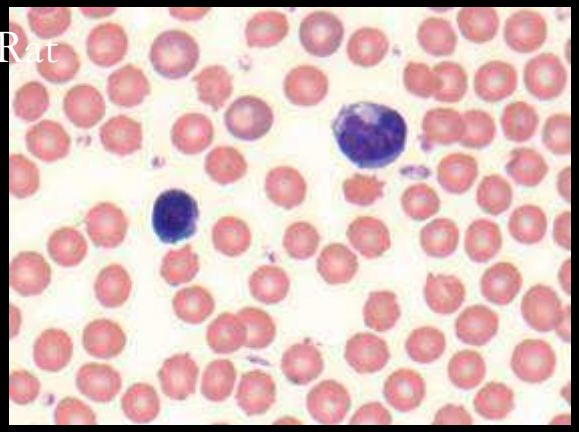
WBC Morphology: Monocytes

- Similar morphology across different species



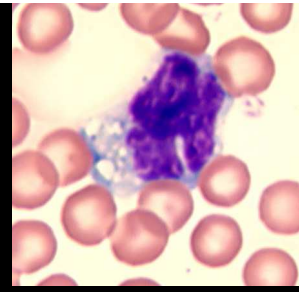


Dog

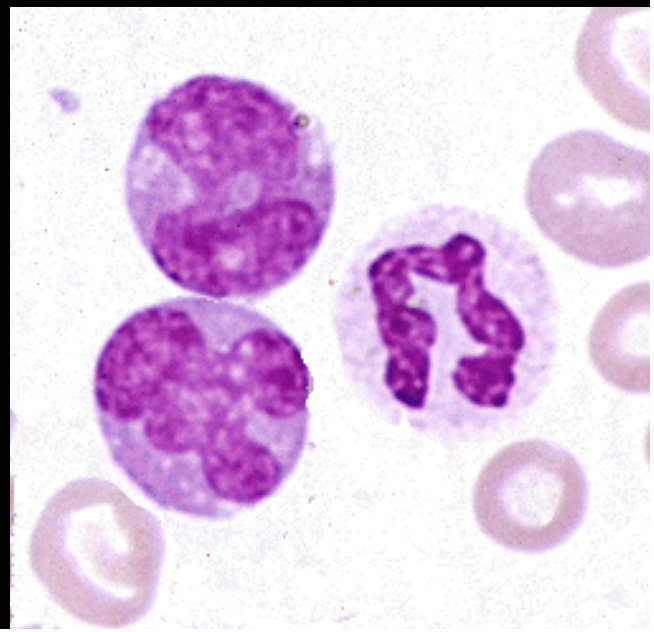


Rat

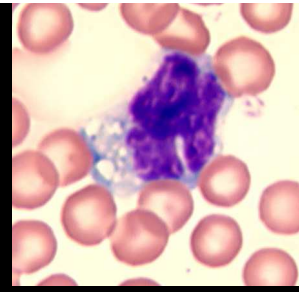
Monocyte Morphology



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



Monocyte Counts

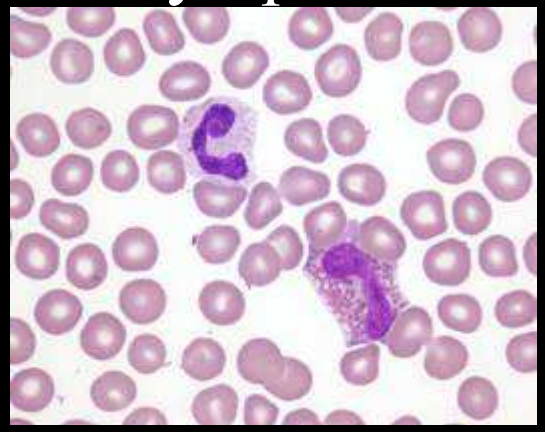


- Increased
  - Corticosteroids (dogs)
  - Inflammation (inconsistent response)
  - Recovery from inflammation
- Decreased
  - Not clinically recognized
  - Observed in conjunction with other cytopenias

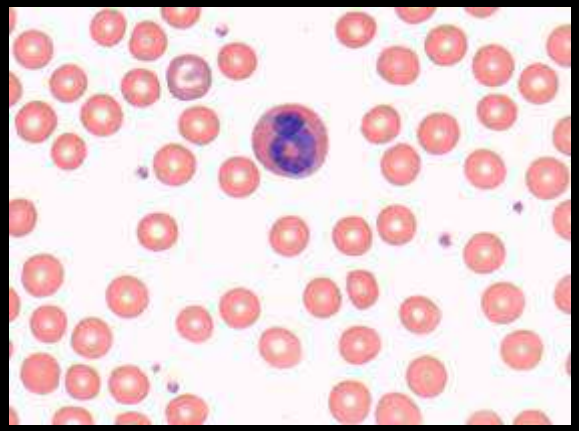
WBC Morphology: Eosinophils

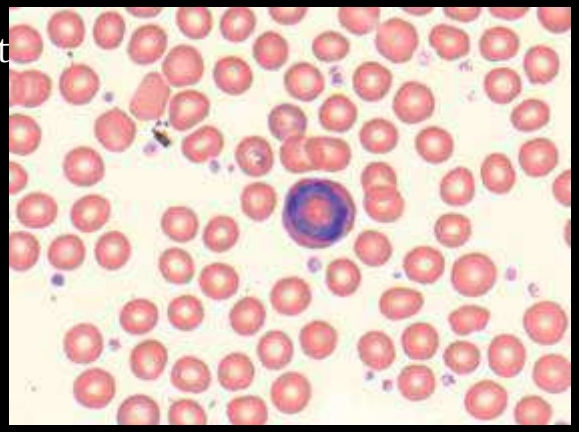
Compared to dogs, rats and mice have…

- Band nucleus
- Finer granules that fill the cytoplasm



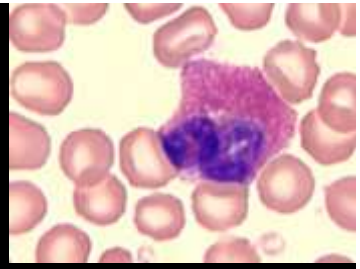
Dog





Rat

Eosinophil Counts

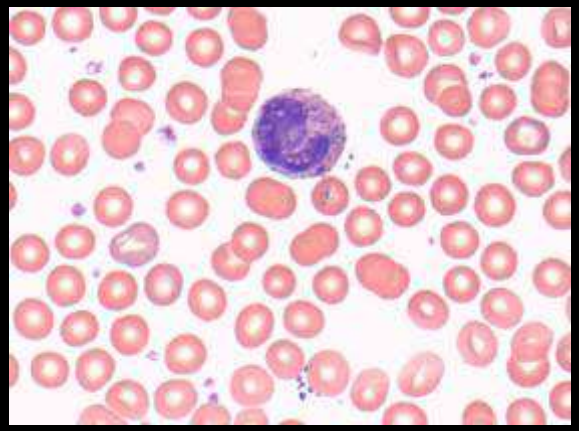


- Increased
  - Almost always mediated by release of IL-5 from T-lymphocytes
    - Inflammatory conditions, hypersensitivities, parasitism (NHPs)
    - Chronic inflammation of tissues rich in mast cells (skin, lung, GI, uterus)
  - Blocked egress
  - Increased production (G-CSF)
  - Mouse platelet clumps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

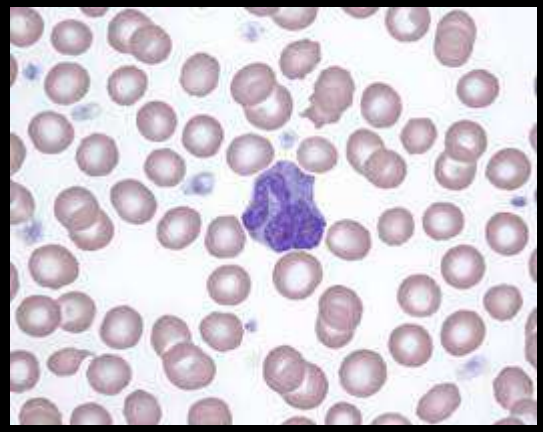
WBC Morphology: Basophils

Compared to dogs, mice and rats have…

- Very few basophils
- Mice DO have basos

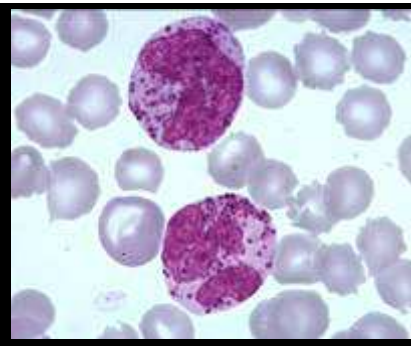


Rat



Dog

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

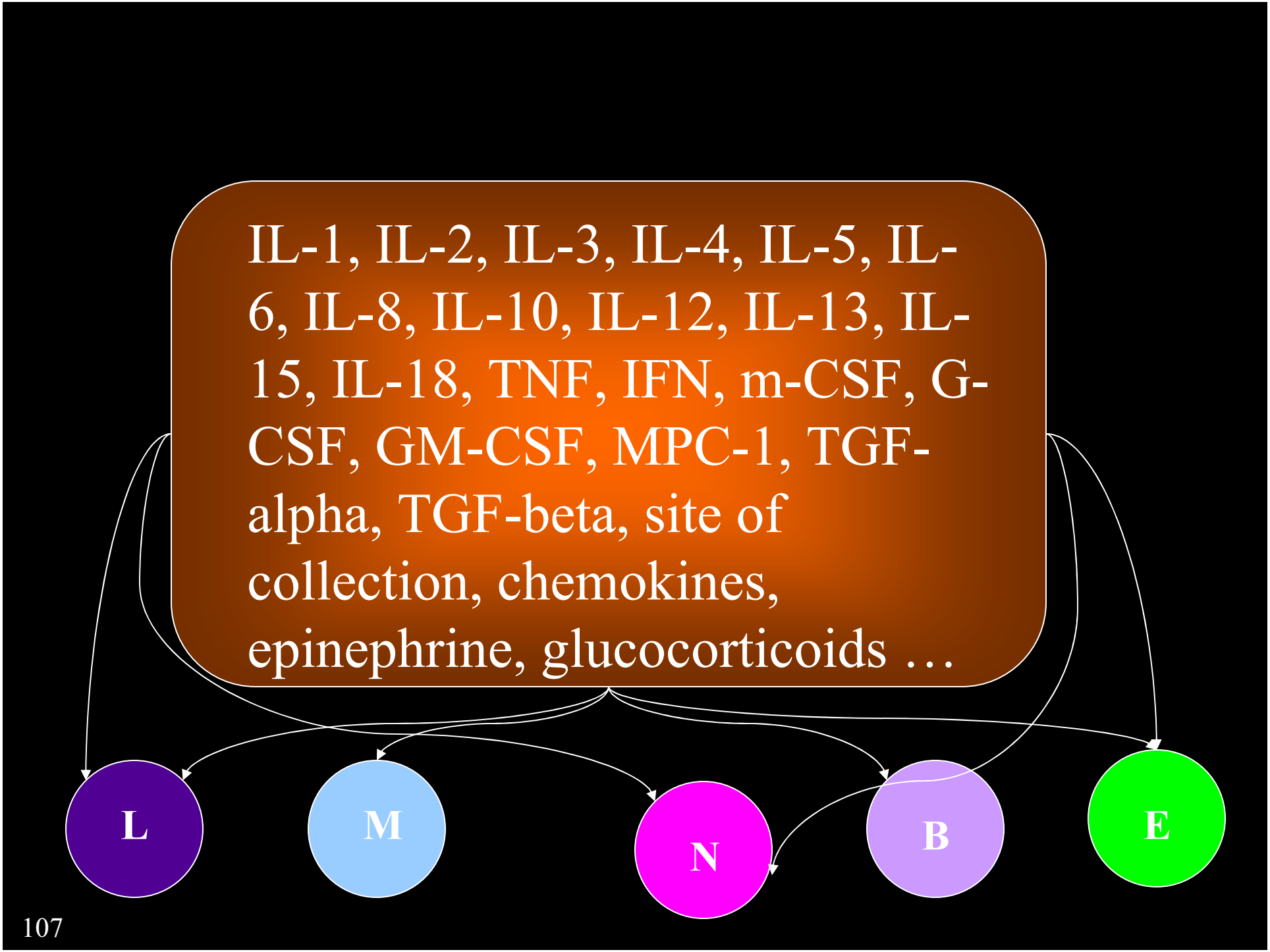
- Normal WBC counts:
  - mouse < rat and rabbit < dog and monkey
- Dependent on sampling site
  - Central counts usually lower than peripheral counts
- Rodent WBCs
  - Mostly lymphocytes
  - Fewer neutrophils; mouse < rat
  - Very few monocytes, eosinophils, and basophils

Review: Increased Leukocytes

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

Review: Decreased Leukocytes

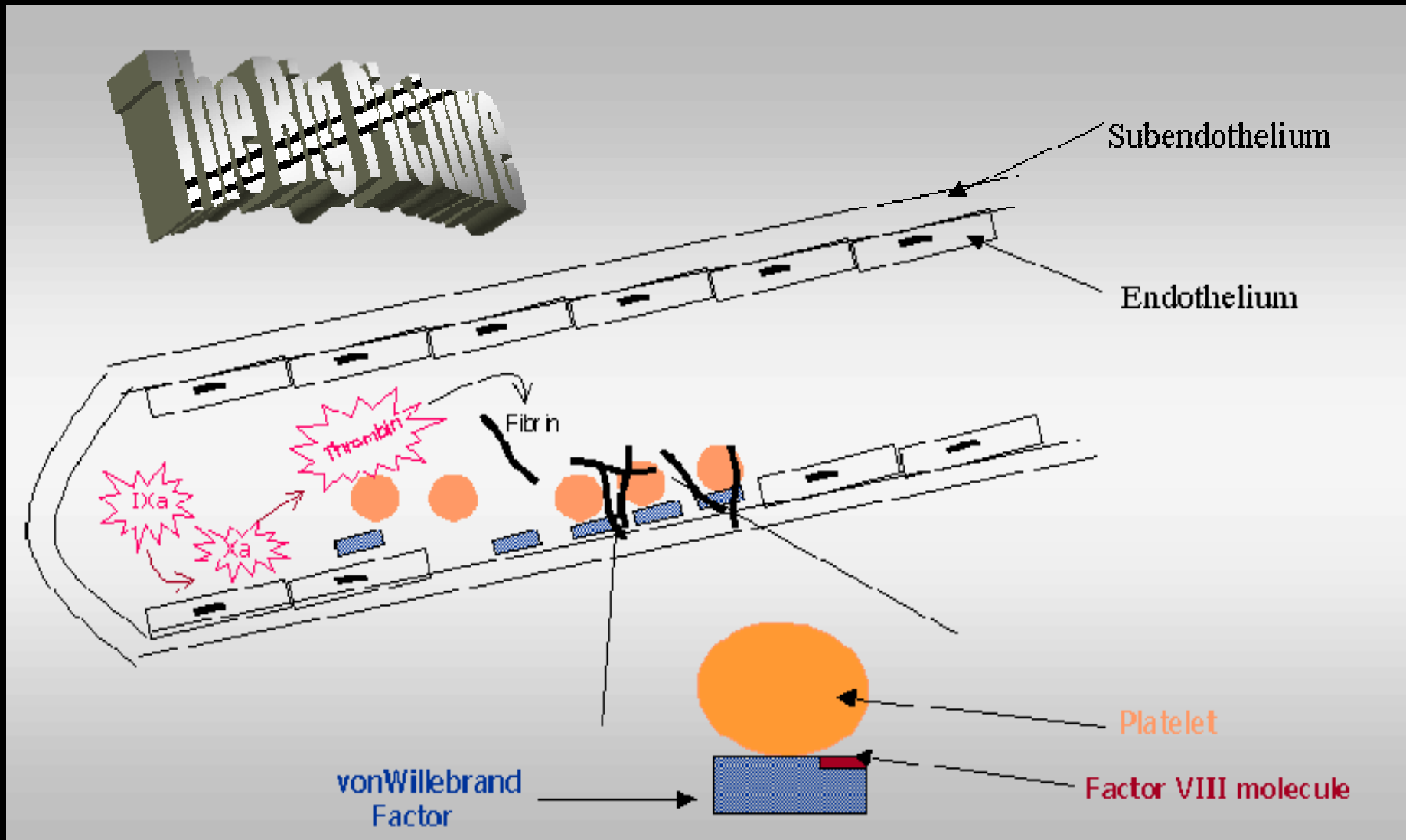
- Decreased bone marrow production
- Glucocorticoid effects
- Peripheral destruction
- Peripheral demand > bone marrow production
- Cytolysis


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 …
L
M
N
B
E

Outline

- Introduction
- Preanalytical considerations
- Erythrocytes
- Leukocytes
- Hemostasis
- Case Examples

Hemostasis

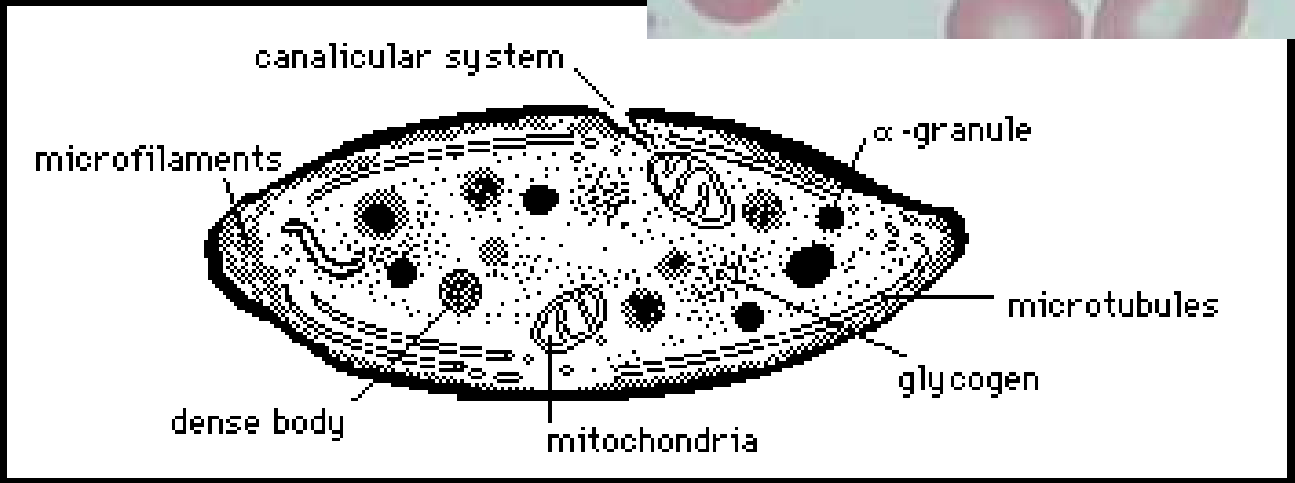


Adapted from Craig C. Kitchens, M.D., lecture given to 2nd year Medical Students, January 1996.

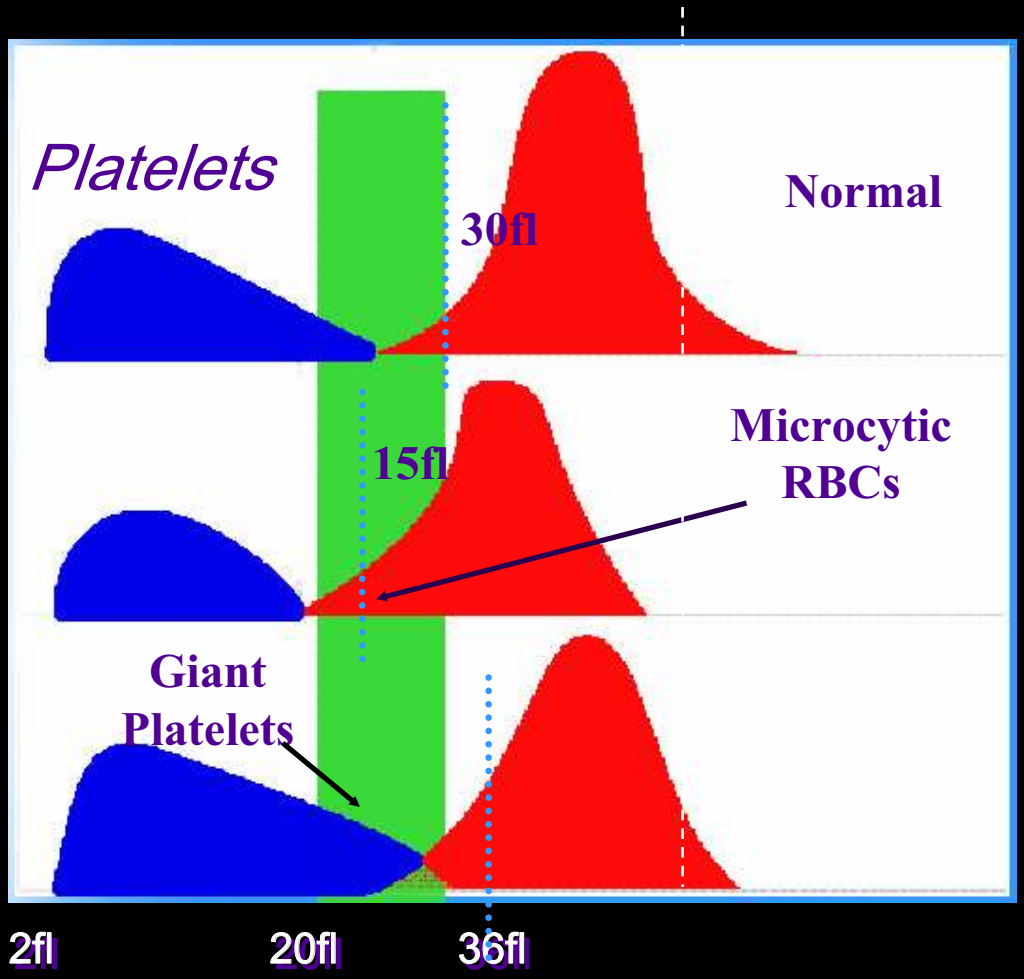
Causes and Symptoms of Hemorrhage

- 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


canalicular system
microfilaments
α-granule
microtubules
glycogen
dense body
mitochondria

Platelet counting/sizing (analogous to RBCs)



Platelet count

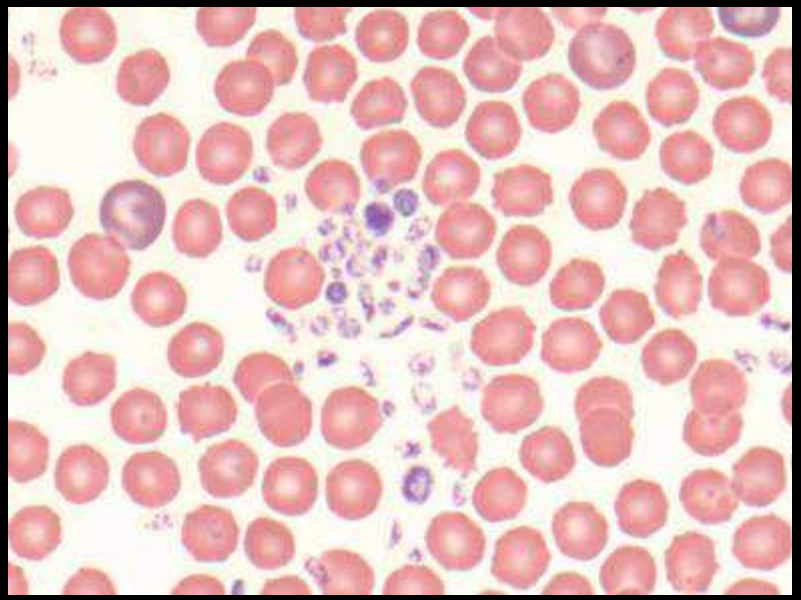
MPV

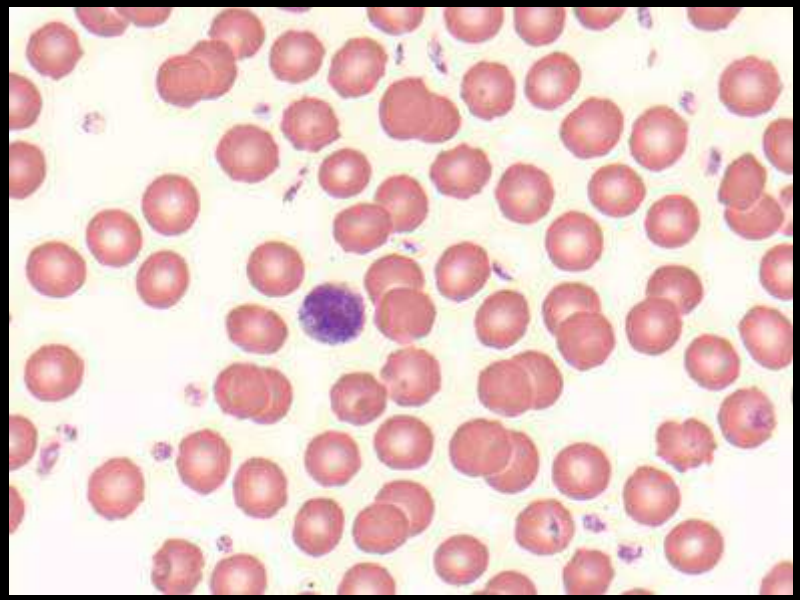
Platelet "crit"

PDW

Platelets

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





Changes in Platelet Size

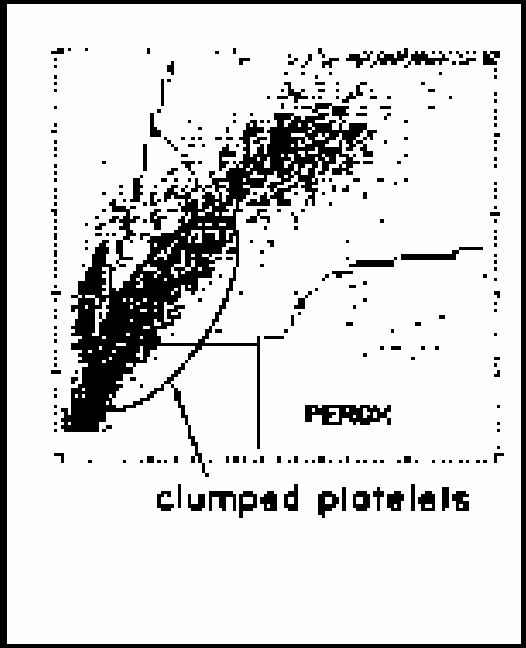
- Large platelets
  - Observed during accelerated production of platelets
- Mean platelet volume (MPV)
  - Follow on an individual animal basis
  - Compare to pretest values as well as concurrent controls
  - Increased with accelerated platelet production

Platelets

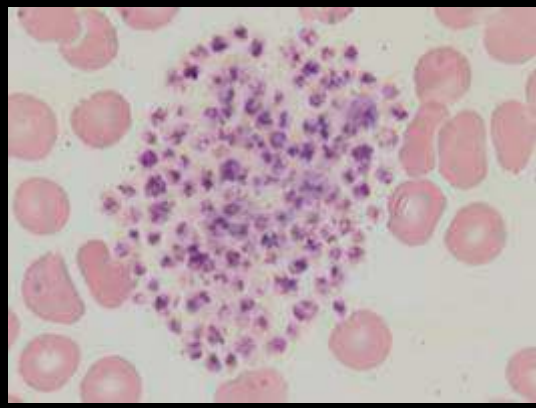
- Platelet counts: rodent >>> non-rodent
- Large platelets more common in rodents
- Increased platelets (sometimes giant platelets)
  - Hemolysis / increased hematopoiesis
  - Also endogenous/exogenous EPO, TPO, other growth factors
- Decreased platelets
  - Platelet clumps; poor technique or sick animal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



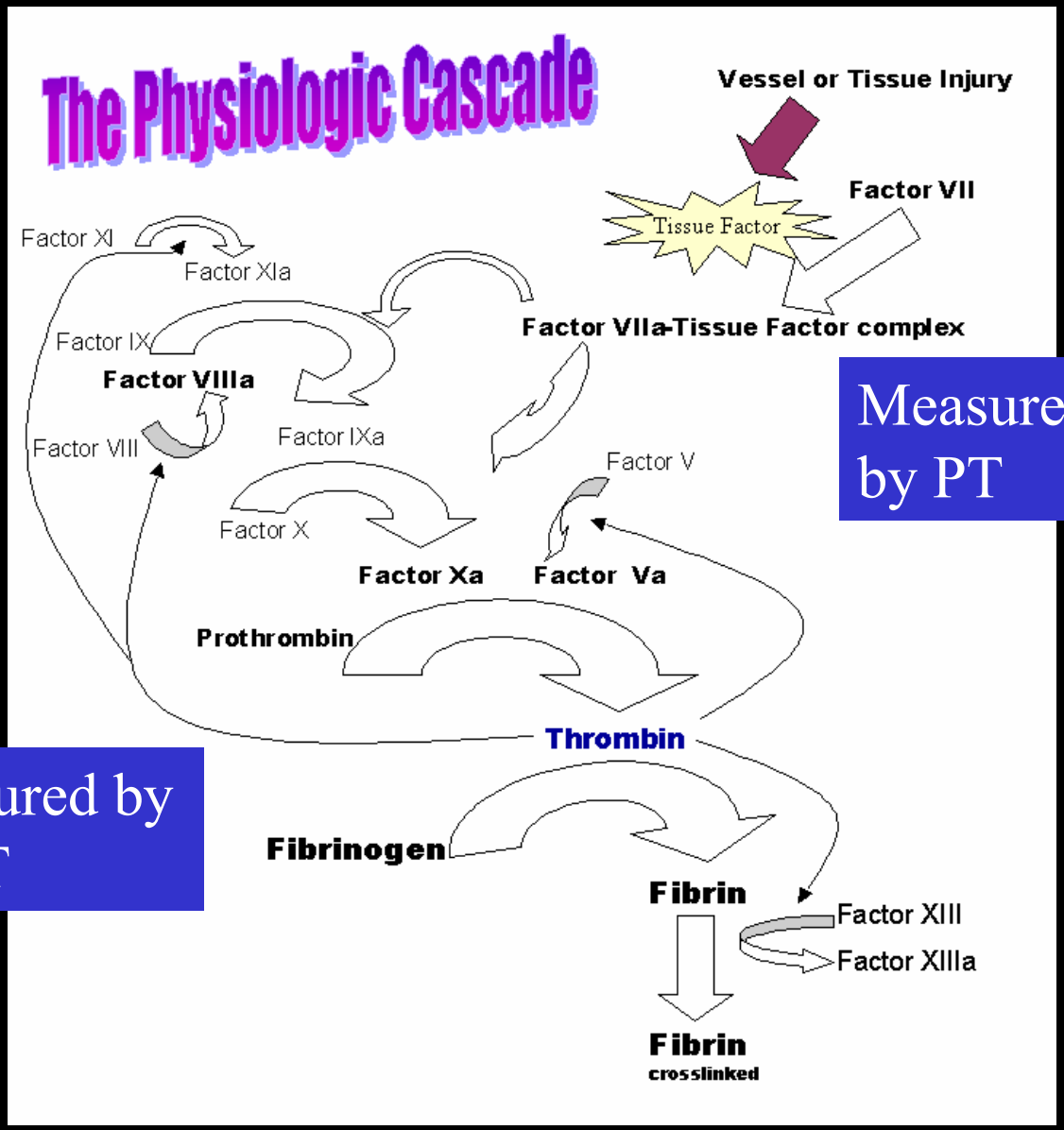
Increased WBC, Eosinophils



Coagulation Parameters

Prothrombin Time,
Activated Partial Thromboplastin Time

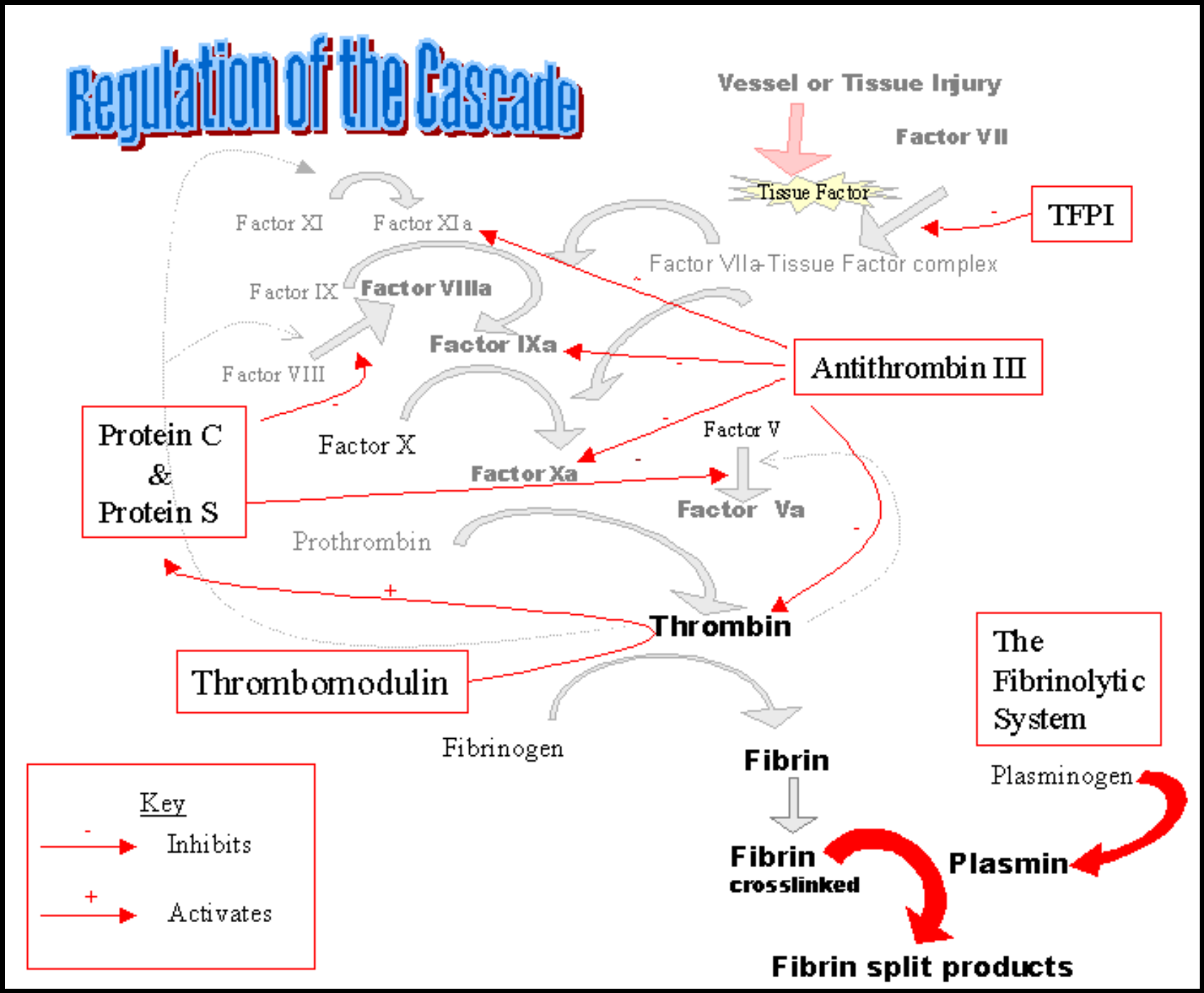
FYI: Coagulation Parameters



Measured by PT

Measured by APTT

http://www.medinfo.ufl.edu/year2/coag/physcasc.html


Regulation of the Cascade
Vessel or Tissue Injury
Factor VII
Tissue Factor
TFPI
Factor XI
Factor XIa
Factor VIIa-Tissue Factor complex
Factor IX
Factor VIIIa
Factor IXa
Antithrombin III
Factor VIII
Protein C
&
Protein S
Factor X
Factor V
Factor Xa
Factor Va
Prothrombin
Thrombin
Thrombomodulin
The
Fibrinolytic
System
Fibrinogen
Fibrin
Plasminogen
Fibrin
crosslinked
Plasmin
Fibrin split products
Key
Inhibits
Activates

PT and APTT: Species Differences

- APTT and PT variable across species

Test (sec)	Human	NHP	Dog	Rat	Mouse
PT	10-12	10-12.1	8.3-10.0	15.1-16.6	11-15
APTT	30-45	15-25	11.5-14.5	12.6-16.3	32-50

- 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
- Preanalytical considerations
- Erythrocytes
- Leukocytes
- Hemostasis
- Case Examples

Predictability of Animal Testing

- Predictability of data from preclinical studies for human hematotoxicity ≥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

Olson H et al. Regul Toxicol Pharmacol. (2000) 32: 56-67. Concordance of the toxicity of pharmaceuticals in humans and in animals.

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

Olson H et al. Regul Toxicol Pharmacol. (2000) 32: 56-67. Concordance of the toxicity of pharmaceuticals in humans and in animals.

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"

Hart JE Pharmac Ther 47: 203-218 (1990)

Human Idiosyncratic Drug Reactions- Hematotoxicity

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

Shenton et al Chemico-Biol Interact 150 (2004) 53-70
Chen Clin Med Res 3 (2005) 102-8
Uetrecht 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

Shenton et al Chemico-Biol Interact 150 (2004) 53-70
Chen Clin Med Res 3 (2005) 102-8
Uetrecht 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 IDRs
  - 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
    - Eythroid 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 asssessed 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