Differentiating between stress and immunotoxicity in toxicology studies

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Caveats and Disclaimers

• Overview
  – Not a comprehensive study of stress effects or identifying immunotoxicity

• Practical advice
  – Routine toxicology studies, routine parameters
  – When should additional (investigative and/or functional) studies be conducted to evaluate the immune system?
  – What are the concerns from human risk and regulatory perspectives?

• References at the end, especially
  – Everds et al
  – ICH guidance document
  – Papers describing examination of lymphoid tissues
Outline

• “Stress” in the context of toxicology studies
• Minimizing stress as a confounding factor
• Effects of stress on immune system endpoints
• Effects on non-immune system endpoints
• Human risk and regulatory considerations
• Differentiating between stress effects and direct immunotoxicity
• What if you suspect direct immunotoxicity?
• Case Study
• Conclusions
The Stress Response

• General Causes (a few examples)
  – Change in environment
  – Perceived or real threat, excitement, starvation
  – Pain, infection
  – Circadian rhythm, waking, feeding, exercise, etc.

• Mild “stress” is a good thing—adaptation mechanism
  – Fight or flight
  – Enhances immune responses
  – Promotes a number of beneficial physiologic processes

• Primary mediators
  – Catecholamines (e.g. epinephrine)
  – Corticosteroids

• Acute Stress
  – Single event

• Chronic Stress (typical of toxicity studies)
  Longer continuous stressor(s) or repeated short stressor(s)
Stress in the context of a toxicology study

• Transportation
• Restraint, handling stress, sample collection, etc.
• Husbandry
  – Social stresses in group housing
    • Incompatible cage-mates
    • Establishing hierarchy
  – Single housing stress for social species
  – Light conditions, feeding regimen
  – Temperature, humidity, bedding, enrichment
• Circadian rhythm (is governed by corticosteroids)
• Administration of test article
  – Taste aversion, emesis, etc.
• Effects of test article
  - Anorexia, weight loss, organ toxicity, physiologic effects, etc.
Minimizing stress as a confounding factor

• Reduce or eliminate causes of stress which can be controlled
  – Acclimation period after transportation
  – Accustom animals to handling; for primates, training is helpful
  – At least two pretests are valuable for primate blood collection
    • Accustoms animal to bleeding; first sample is most likely to show epinephrine-related response
    – Use well-trained personnel, preferably use same individuals consistently

• Optimize husbandry conditions
• Distribute different ages of animals across dose groups
• Keep in mind circadian rhythm effects
  – Dose, collect samples, conduct procedures at the same time of day each time conducted
    • Minimizes sample variability as well as stress
• Randomize sample collection and processing
• Consistency is key—acclimation → habituation, which minimizes stress hormone release and effects
Stress and the immune system (or “is stress an immunotoxicant?”)

• Catecholamines from adrenal medulla
  – Increase in all blood cell types in circulation (transient) due to demargination and mobilization from spleen
  – Influence lymphocyte development and function
  – Influence myelopoiesis and lymphopoiesis in various ways
Stress and the immune system (or “is stress an immunotoxicant?”)

- Corticosteroids from adrenal cortex
  - Neutrophils: increased release of mature segs from bone marrow, retention in circulation, survival
  - Increased apoptosis of eosinophils
  - Lymphocytes: basal CCS levels promote survival, differentiation, Ab production; but with stress:
    - Decreased in circulation due to redistribution
    - Decreased cellularity of lymphoid tissues
      - diminished proliferation
      - apoptosis
    - Immature CD4+/CD8+ thymic lymphocytes more sensitive than single +, more mature cells
- In reality, catecholamines and corticosteroids both play a role and the systems are intertwined
Stress and the immune system (or “is stress an immunotoxicant?”)

- IL-6, IL-1 mediated induction of acute phase response
- Suppression of cell mediated immunity
- Enhancement of pro-inflammatory, Ab-mediated responses
- Positive and negative feedback loops involving glucocorticoids, catecholamines, and cytokines
- Humans under chronic stress have impaired immunity and immune responses to challenge
Effects of stress on immune system endpoints

- Hematology (effects seen before organ weight, histopath findings)
  - Physiologic (epinephrine) leukocytosis
    - Increases in all WBCs proportionately
    - Most common non test article-related hematology finding in primate studies
      - Especially first bleeding interval, transient
  - Stress (corticosteroid) leukogram
    - Lymphopenia=most consistently seen part of the leukogram
    - Neutrophilia with no left shift
    - Eosinopenia
      - Not always appreciable due to low normal numbers
    - Monocytosis
      - More variable, species-dependent
Effects of stress on immune system endpoints

- Organ weight decreases in lymphoid tissues (effects seen before histopath findings)
- Histopath findings
  - Lymphoid depletion
    - Apoptosis
    - Cell debris, tingible-body macrophages: “starry sky” in acute stress
- Tissues affected in order of severity or chronicity of stress
  1. Thymus: most susceptible due to population of immature, especially double positive cells
    - Cortex frequently decreased in size
  2. Spleen
    - Decreases in white pulp, decreased cellularity of MZ and PALS follicles
  3. Lymph nodes
  4. Bone marrow
Thymus
Both images at original magnification of 2x

Control

Depletion

Courtesy of Mike Mirsky
Acute stress
Lymphocyte necrosis or lymphocytolysis

Thymus - acute hemorrhage at the cortex-modularly junction

Thymus – necrotic lymphocyte debris

Courtesy of Xiantang Li
Subacute stress

Mesenteric lymph node—necrotic lymphocyte debris

Courtesy of Xiantang Li
Stress – lymphoid depletion & atrophy
Differentiate normal, involution and atrophy

Spleen

Normal - lymphoid

Lymphoid depletion/atrophy

Spleen

Courtesy of Xiantang Li
Effects of stress on non-immune system endpoints: additional evidence in WOE review

• None of these findings is specific for stress
• For all findings, keep in mind the possibility of direct test article-related effects in your weight of evidence review
• Some findings may be countered or negated by other factors
• You may not see the entire spectrum of findings in a given animal
• Seeing these effects supports “stress” conclusion, but not seeing them doesn’t rule it out

For a comprehensive review, please see Everds et al 2013
Effects of stress on non-immune system endpoints: additional evidence in WOE review

In rough order of helpfulness

• Anorexia, weight loss, decreased food consumption
• Adrenal hypertrophy
  – adrenal medullary and cortical (esp. zona fascicularis) hypertrophy in chronic stress
  – increased pheochromocytoma incidence in some species)
• Increased pituitary weight, hypertrophy and proliferation of corticotrophs which secrete ACTH
• Hyperglycemia
  – Epinephrine-induced gluconeogenesis, glycogenolysis
  – Corticosteroid-induced gluconeogenesis, insulin resistance
  – Glucagon glycogenolysis and gluconeogenesis
• Induction of corticosteroid-specific alkaline phosphatase isoenzyme in dogs
• Increased red blood cell mass in circulation (epinephrine →release from bone marrow, spleen)
• Increased cortical thickness
• **Zona fasciculata usually enlarged**
  – Cell enlarged
  – Abundant eosinophilic cytoplasm and decreased vacuoles
  – Started from outer to inner zona
• **Zona glomerulosa and zona reticularis cells may be enlarged**
Human risk and regulatory considerations

• Why is it important to differentiate between stress and direct immunotoxicity?
  – Stress effects due to overt toxicity or experimental conditions at high doses
    • Secondary/indirect effect of test article administration
    • Unlikely to suggest human risk providing there is a good safety margin
    • No need to pursue mechanism or derisk immunotoxicity
    • Other toxicities likely to be of greater importance
Human risk and regulatory considerations

• Why is it important to differentiate between stress and direct immunotoxicity?
  – Direct immunotoxicity
    • May suggest human risk potential
    • Need to determine mechanism, functional consequences
    • May be unacceptable depending on risk/benefit assessment
      – indication, patient population, duration of exposure, therapeutic index, etc.
Critical to characterize lymphoid findings appropriately

• Sensitivity of “stress” call to regulators
  – Guidances specifically indicate concern
  – Historically contributed to lack of confidence in adequacy of pathologic diagnoses re immune system

• Importance to program
  – Writing off lymphoid findings to “stress” may miss an immunotoxic effect
  – Over-calling immunotoxicity may result in
    • delays to program
    • additional resource commitment to conduct functional assays
    • unwarranted regulatory or clinician anxiety
    • discontinuation of program
Human risk and regulatory considerations

• ICH S8 guidance
  • weight of evidence approach to identify immunotoxicity
  • heavy reliance on clinical pathology, organ weights, histopathology to identify unexpected/unintended immunosuppression
  • scientific rationale for choosing follow-on studies
  • “stress” call should have strong justification
Findings in STS which suggest immunotoxicity

• Two main principles
  – Effects on cells or tissues typically involved in immune responses
  – Evidence of infection
Findings in STS which suggest immunotoxicity: weight of evidence

- Clinical Signs
- Clinical Pathology Parameters
  - Hematology
    - RBC, platelets
    - WBC and differential, immunophenotyping when conducted
  - Serum Chemistry
    - globulins
  - Urinalysis
- Anatomic Pathology Findings
  - Bone marrow
  - Lymphoid tissue
  - Presence of inflammation, organisms in other tissues
Distinguishing between stress effects and direct immunotoxicity

• Immune system effects seen independently of stress effects
  – Effects at doses lower than overt toxicity findings
  – Effects not typical of stress-related immune system effects
  – Effects “out of order”, e.g. lymphoid depletion in peripheral node in absence of thymus changes
  – No evidence of stress in any dose group

➢ Presumed immunotoxicity
  – Determine mechanism, functional effects, human risk potential
## Hematology: Differentiating stress from immunotoxicity

<table>
<thead>
<tr>
<th></th>
<th>Stress Leukogram (corticosteroid)</th>
<th>Immunotoxicity: Inflammation/infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Typical picture peripheral blood</td>
<td>↓Lys, eos ↑neuts, monos Neutrophils are mature</td>
<td>↑neuts, monos ↑Lys (rodents) ↓Lys other species Immature neutrophils, toxic change</td>
</tr>
<tr>
<td>Possible corroborative pathology data</td>
<td>Lymphoid atrophy/depletion Adrenal cortical hypertrophy Hyperglycemia</td>
<td>Inflammation in tissues Organisms Urinalysis suggestive of infection Acute phase response</td>
</tr>
<tr>
<td>Supportive study data</td>
<td>Overt organ toxicity Weight loss, anorexia Deaths in other animals in dose group At or exceeded maximum tolerated dose</td>
<td>Clinical signs of infection, fever, anorexia, weight loss</td>
</tr>
</tbody>
</table>
Neutrophilic “left shift” with toxic change

Immature neutrophils with toxic change

Normal canine basophil and neutrophil
Circulating neutrophil with phagocytosed bacteria (cocci)
## Pathology: Differentiating stress from immunotoxicity

<table>
<thead>
<tr>
<th></th>
<th>Stress</th>
<th>Immunotoxicity: Immunosuppression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphoid depletion</td>
<td>Thymus is involved; may be other tissues as well</td>
<td>If no thymus findings, likely to be direct effect; thymus effect could still be direct immunotox</td>
</tr>
<tr>
<td>in one or more tissues</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Possible corroborative pathology data</td>
<td>Adrenal cortical hypertrophy Hyperglycemia Stress leukogram</td>
<td>Infection, inflammation, Pancytopenia Bone marrow suppression Acute phase response Hypoglobulinemia Severe lymphopenia Inflammatory leukogram</td>
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Some words of caution

- Some findings attributed to “stress” may be effects of the stressor
  - e.g. anorexia association with bone marrow effects, diminished RBC production
    - Anorexia is a symptom of stress
    - Diminished nutrient intake may result in bone marrow suppression
    - Stress doesn’t necessarily directly cause suppression of bone marrow

- Direct toxic effects on the immune system can cause stress
  → stress findings superimposed on immunotoxicity findings
Why not just measure corticosteroids, ACTH, epinephrine, etc.?

- It does not add value to measure stress hormones in a toxicology study
- Results can be difficult to interpret and can confound interpretation
  - Variability over time and between individuals
  - Influence of other factors
  - Blood collection itself induces a stress response
  - Blood values at a given timepoint may not be increased over normal variability in chronic stress with habituation
  - Difficult to distinguish between “good” stress hormone levels and excessive levels at a given timepoint
A few examples of follow-ups to findings which cannot be attributed to stress

Should always be driven by the specific findings

- If no particular mechanism is suggested, first tier might be Tcell Dependent Antigen Response (TDAR)

- Effects on lymphoid tissues
  - Lymphocyte phenotyping (immunohistochemistry in tissues or flow cytometry of peripheral blood, tissue)
  - TDAR, NK, B- and T-cell proliferation

- Evidence of infection
  - In vitro or ex vivo
    - microbicidal activity; yeast, bacteria, and/or bead phagocytosis (macrophage, neutrophil)
    - Calcium mobilization, chemotaxis, adhesion molecule assessment, oxidative burst, complement activation, cytokine evaluation

DTH, TDAR, NK, B- and T-cell proliferation
Case Report

- PDE4 inhibitor, SCH 351591
- Development discontinued because of vasculopathy
- Several immune parameters were affected during standard toxicity studies

*Losco et al. The toxicity of SCH 351591, a novel phosphodiesterase-4 inhibitor, in cynomolgus monkeys. Toxicologic Pathology, 32:295-308, 2004
Immunotoxicity-related findings

• Clinical signs
  – Hypothermia, dehydration, lethargy, rhinorrhea, swollen foot

• Clinical pathology
  – Inflammatory leukograms (neutrophilia, left shift, toxic change), azotemia

• Gross and microscopic necropsy
  – Subcutaneous abscess with bacteria, inflammation, dissemination to heart, kidneys, brain
  – Abscess in muscle of neck, bacterial bronchopneumonia with adhesions to thoracic cavity, pericardium; dissemination to brain and heart
  – Thymic atrophy
Hematology Findings

- WBC $17.2 \times 10^3/\mu L$ (pretest 10.1, 9.1)
  - Lymphocytes 1.0 (pretest 4.9, 5.3)
  - Neutrophils 10.1 (pretest 3.6, 2.1)
  - Bands 4.1

- “Toxic change” in neutrophils:
  - Cytoplasmic basophilia 2+
  - Döhle bodies 2+
  - Vacuolization 1+

Neutrophilia with a left shift, toxic change, lymphopenia: Inflammatory leukogram with stress component
Monkey lung, 20X H&E
Monkey lung, 400X gram stain
Immune function assays

• Even though there was obvious stress, the presence of infection suggested immunosuppression

• Immune function assays were conducted
  – Leukocyte function
  – Lymphocyte proliferation
  – Flow cytometry (immunophenotyping of lymphocytes)
  – If development had progressed, TDAR and NK assays would have been conducted
Noteworthy results of leukocyte function assays and lymphocyte evaluation

• T cell proliferation impairment
  – SCH 351591 had a direct effect on T lymphocyte proliferation
  – Findings in the thymus were most likely a direct effect of test article and could not be attributed to stress alone
Differentiating between stress effects and immunotoxicity: Summary

• It is usually possible to sort out if immune system findings are due to stress or direct effects on the immune system, within the context of the study with standard endpoints

• Weight of evidence
  – Clinical signs, clinical pathology, organ weights and histopath, expected effects of drug/chemical
  – Other indications of overt toxicity, stress
  – Magnitude of response compared with stressor
  – Alternative interpretations for findings?
    • put the puzzle pieces together
  – Indications of immunotoxicity, especially immunosuppression?
References of interest

Acknowledgments

- Jay Fine
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