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# Chapter 6

# Clinical Chemistry of the Laboratory Mouse

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| | | |
|---|---|---|
| I. | Introduction | 173 |
| | A. The Mouse in Biomedical Research | 173 |
| | B. New Techniques and Instrumentation | 174 |
| | C. Effect of Drug Development Regulation | 174 |
| II. | Methods of Analysis and Instrumentation | 175 |
| | A. Introduction | 175 |
| | B. The Changing Nature of Clinical Chemistry | 175 |
| | C. Immunologic Methods to Detect Mouse Analytes | 175 |
| | D. Multiplex Technology | 176 |
| | E. Sources of Information | 179 |
| III. | Sampling | 179 |
| IV. | Reference Ranges | 180 |
| | A. Introduction | 180 |
| | B. Variability | 180 |
| | C. Quality Assurance | 182 |
| | D. Statistics | 182 |
| V. | Specific Tests | 182 |
| | A. Introduction | 182 |
| | B. Glucose and Carbohydrate Metabolism | 182 |
| | 1. Glucose and Its Measurement | 182 |
| | 2. Diabetes | 183 |
| | 3. Relationships Between Obesity and Diabetes | 184 |
| | 4. Measurement of Hormones Associated with Carbohydrate Metabolism and Food Intake | 185 |
| | 5. Measurement of Adipokines | 185 |
| | 6. Other Analytes Involved with Adipogenesis and Insulin Resistance | 185 |
| | C. Lipid Metabolism | 185 |
| | 1. Types and Functions | 185 |
| | 2. Atherosclerosis | 186 |
| | 3. Measurement | 187 |
| | a. Serum Cholesterol and Triglycerides | 187 |
| | b. HDL, LDL, IDL, and VLDL | 188 |




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c. Mouse Apoproteins .................................. 188
d. Other Analytes Associated with Lipid Metabolism and Atherosclerosis in Mice ............................ 188
D. Immunoglobulins and Immune Function ........................ 188
1. Immunoglobulins (Ig) .................................. 189
2. Complement .......................................... 190
3. Circulating Immune Complexes (CIC) ...................... 191
4. Autoantibodies ....................................... 191
5. Cytokines and Chemokines ............................... 191
a. Interleukins (IL) .................................... 191
i. Interleukin-1 (IL-1) .............................. 191
ii. Interleukin-2 (IL-2) .............................. 192
iii. Interleukin-3 (IL-3) .............................. 192
iv. Interleukin-4 (IL-4) .............................. 192
v. Interleukin-5 (IL-5) .............................. 192
vi. Interleukin-6 (IL-6) .............................. 192
vii. Interleukin-7 (IL-7) .............................. 192
viii. Interleukin-8 (IL-8) .............................. 192
ix. Interleukin-10 (IL-10) ............................ 192
x. Interleukin-11 (IL-11) ............................ 192
xi. Interleukin-12 (IL-12) ............................ 193
xii. Interleukin-13 (IL-13) ............................ 193
xiii. Interleukin-17 (IL-17) ............................ 193
xiv. Interleukin-18 (IL-18) ............................ 193
xv. Interleukin-20 (IL-20) and Interleukin-21 (IL-21) ...... 193
b. The Transforming Growth Factor–β Superfamily (TGF-βSF) of Cytokines .............................. 193
c. The Tumor Necrosis Factor Superfamily (TNFSF) .......... 194
i. Tumor Necrosis Factor–α (TNF-α) ................. 194
ii. Tumor Necrosis Factor–β (TNF-β) ................. 194
iii. CD40L ...................................... 194
iv. CD30L ...................................... 194
v. Fas Ligand (FasL) .............................. 194
vi. TNF-Related Activation-Induced Cytokines (TRANCE) ................................... 194
vii. TNF-Receptor Superfamily (TNFRSF) ............. 194
d. Interferons ......................................... 195
i. Interferon-α (IFN-α) ........................... 195
ii. Interferon-β (IFN-β) ............................ 195
iii. Interferon-γ (IFN- γ) ............................ 195
e. Chemokines ......................................... 195
6. Arachidonic Acid Metabolites ............................ 196
E. Enzymes ................................................ 198
1. Alkaline Phosphatase (AP) ............................... 198
2. Alanine Aminotransferase (ALT) .......................... 198
3. Aspartate Aminotransferase (AST) ......................... 198
4. Lactate Dehydrogenase (LDH) ............................ 199
5. Ornithine Transcarbamylase (OTC) ........................ 199
6. Creatine Kinase (CK) .................................. 199
7. Aldolase ............................................. 200
8. Sorbitol Dehydrogenase (SDH) ........................... 200
9. Amylase ............................................. 200
10. Other Enzymes ....................................... 200
F. Endocrine Hormones ...................................... 200
1. Corticosterone and Adrenocorticotropic Hormone (ACTH) ..... 200
2. Luteinizing Hormone (LH) ............................... 201
3. Follicle-Stimulating Hormone (FSH) ....................... 201
4. Androgens ........................................... 202
5. Estradiol ($E_2$) ........................................ 202
6. Progesterone ......................................... 203
7. Prolactin (PRL) ....................................... 203
8. Oxytocin ............................................ 203
9. Vasopressin (AVP) .................................... 204
10. Thyroid-Stimulating Hormone (TSH) ...................... 204

11. Thyroxine ($T_4$) and Triiodothyronine ($T_3$) .......... 204
12. Parathyroid Hormone (PTH) .......... 205
13. Other Hormones .......... 205
G. Liver Function Tests .......... 205
1. Enzymes .......... 205
2. Bilirubin .......... 205
3. Bile acids .......... 206
4. Serum Proteins .......... 206
5. Dye Excretion .......... 206
H. Kidney Function Tests .......... 206
1. Urea Nitrogen .......... 206
2. Creatinine .......... 207
3. Urinalysis .......... 207
I. Electrolytes .......... 207
1. Sodium, Potassium, Chloride, and Phosphorus .......... 207
2. Calcium .......... 207
J. Other Proteins .......... 208
References .......... 208

# I. INTRODUCTION

It has been more than 20 years since publication of the first edition of *The Mouse in Biomedical Research,* and since that time new emphasis has been placed on the mouse as a model for the pathophysiology and treatment of human diseases. During this time, the frontier of clinical chemistry in the mouse has advanced and expanded because of two major events: the increasing reliance on mice in biomedical research, and increasing availability of practical yet sophisticated techniques and instrumentations that have allowed for the detection of a wider variety of biomarkers of disease. The progression of these two events has been partially driven by the increasing regulatory demands related to safety/toxicity assessment of novel drug development.

Abbreviations used in this chapter are summarized in Appendix 6-1.

## A. The Mouse in Biomedical Research

For the last quarter century, mice have been the most frequently used mammal in biomedical research, rising from 61% of all mammals used in 1983 to 71% in 1993 (National Center for Research Resources 1997). The nature of their use has changed dramatically during this time. During the first half of the 20th century, a great deal of emphasis was placed on the development of inbred strains. The availability of inbred strains has led to major breakthroughs in cancer, biology, and immunology (Quimby 2002). By 1970 there were approximately 250 inbred strains available. In addition, outbred stocks continue to be utilized in a wide variety of studies but particularly in the fields of toxicology and pharmacology. During the second half of the 20th century, investigators developed congenic lines and recombinant inbred strains, each having an impact on elucidating the role of individual genes and assigning new traits to linkage groups respectively (Paigen 2003a). However, beginning in the early 1980s scientists began to genetically engineer mice by either adding a new gene (transgenic) or deleting a normal mouse gene (knockout, KO) (Paigen 2003b). The power of these models to elucidate the genetic basis of disease cannot be overemphasized. Over the past 15 years, publications citing transgenic and KO mice have increased exponentially and at the time of this writing the Mutant Mouse Resource of the Jackson Laboratory listed more than 10,000 such unique lines. During this same period the results of both the human and mouse genome projects were published (International Human Genome Sequencing Consortium 2001; Mouse Genome Sequencing Consortium 2002). This provided complete nucleotide sequences for each genome allowing investigators to quickly develop the equivalent murine model for many of the inherited human diseases. Transgenic and KO mice have helped clarify disease pathogenesis in virtually every area of medicine and often elucidated biochemical pathways, previously unknown, which are now subject to testing and quantification. As a result, clinical chemistry in the mouse has grown from evaluation of 15–20 analytes found in plasma (or urine), to hundreds of biomarkers that can monitor disease status at the cellular level.

Due to the massive amount of new information characterizing each of the standard strains and mutant lines of mice, a Mouse Phenome Database has been developed to manage data and provide researchers with the ability to explore raw phenotypic data (including clinical chemistry) and summary analyses. The resource allows investigators to select the appropriate murine model for physiology testing, drug discovery, toxicology studies, mutagenesis, modeling human disease, quantitative trait loci (QTL) analyses, identification of new genes, and unraveling the influence of environment on genotype (Bogue and Grubb 2004).

### B. New Techniques and Instrumentation

By far, the most important new technology in the rapidly growing and changing field of clinical chemistry is the discipline of proteomics. Proteomics combines the disciplines of molecular biology, biochemistry, and genetics to the analysis of the structure, function, and interactions of proteins produced by the genes of a particular cell, tissue, or organism.

The field of proteomics has grown mainly due to the development of new instruments that are based on sophisticated techniques, yet amenable to practical implementation. For example, the development of surface-enhanced laser desorption/ionization (SELDI) platform time-of-flight (TOF) mass spectroscopy (MS) allowed Petricoin *et al.* (2002a) to identify five peptides in the sera of women with ovarian cancer that were not found in women without ovarian cancer, even though the structure and function of some of these peptides were not actually known. Building on these findings, Zhang *et al.* (2004) combined the SELDI-TOF MS with protein separation procedures to develop a multianalyte immunoassay for rapidly screening potential cancer patients. This process of identifying proteins (biomarkers) that are predictive for a disease is known as plasma protein profiling. Similar technology has been used to develop plasma protein profiles for neoplastic conditions in humans, such as prostate cancer (Cazares *et al.* 2002; Petricoin *et al.* 2002b; Qu *et al.* 2002) and bladder cancer (Adam *et al.* 2001; Vlahou *et al.* 2001), as well as a variety of non-neoplastic conditions, such as ischemic versus hemorrhagic stroke (Allard *et al.* 2004), severe acute respiratory syndrome (Kang *et al.* 2005), Alzheimer's disease (Carrette *et al.* 2003), and Creutzfeldt-Jakob disease (Sanchez *et al.* 2004).

Plasma protein profiling has also begun in mouse models. Xiang *et al.* (2004) used a combination of two-dimensional gel electrophoresis (2D-GE) and matrix-assisted laser desorption/ionization (MALDI) TOF MS to quantify serum protein profiles of C57BL/6 mice harboring the Lewis carcinoma with and without treatment using acetazolamide. They found upregulation of many peptides associated with tumor growth and metastasis, and many of these same peptides were modified during treatment. Two specific targets of acetazolamide antitumor activity were subsequently identified as histone H2B and CROC1 (a UBC-like peptide). Park *et al.* (2004) used similar technology to determine plasma protein profiles for high (C57BL/6) and low (C3H) atherosclerosis prone strains of mice on normal or atherogenic diets. They identified 30 proteins in which the levels had changed after eating an atherogenic diet. Of these, 14 were differentially changed in C57BL/6 mice and an additional 16 were changed in both strains. In addition, 28 proteins were differentially expressed between the two strains regardless of diets. Four of these proteins were upregulated in C57BL/6 and 11 were upregulated in C3H. Nine of those protein markers were definitively identified and their roles in the pathogenesis of atherosclerosis discussed in the "Atherosclerosis" section.

Plasma protein profiling in mice has the potential to become an important discovery tool for translational research, particularly in identifying cancer-associated plasma biomarkers in humans. For example, Juan *et al.* (2004) also used 2D-GE and MALDI TOF MS to identify plasma biomarkers in BALB/c-nude mice harboring human xenotransplanted tumors. In mice bearing a human stomach cancer cell line, serum amyloid A (SAA) was elevated. The authors then went back to screen the sera of human stomach cancer patients and were able to demonstrate that, when compared to controls, humans with stomach cancer also had significantly elevated levels of SAA.

All of these examples illustrate the power of proteomics. Proteomic pattern diagnostics offers a means to look at molecular diagnostic information in human or mouse serum, without preconceived assumptions about the existence or identity of the biomarkers. This allows for the development of sensitive and specific peptide assays for identified biomarkers of disease. Practical applications of proteomics have been facilitated by the creation of instrumentation that can analyze multiple analytes from small sample volumes with fast through-put (such as multiplex technology, which are discussed in the "Multiplex Technology" section).

In an attempt to manage all the experimental data arising from the fields of proteomics, metabolomics and transcriptomics (gene array), the Chemical Effects in Biological Systems (CEBs) Knowledge base was developed (Xirasagar *et al.* 2004). This is a useful, searchable database that integrates experimental findings from all three disciplines, by biosource identification (animal, test article, genotype, and investigator). By making the cross correlation from data arising from three different streams it is hoped that combination groups of predictive markers for disease and toxicity will emerge.

### C. Effect of Drug Development Regulation

The cost of bringing a new drug to market is estimated at $0.8–1.7 billion (Food and Drug Administration 2004), of which a large proportion is spent on safety/toxicity assessments during the preclinical phase of the development of a drug. Although expensive, safety/toxicity regulations are necessary, as illustrated by the fact that a new drug entering Phase I clinical trials has only an 8% chance of reaching the marketplace due to toxicity issues. The ability to decrease the costs of drug development due to safety/toxicity issues is becoming increasingly reliant on the use of the laboratory mouse as a model for human disease and the identification of sensitive biomarkers for toxicity and disease.

The underlying basis of using laboratory mice as models for human disease was demonstrated by Everett and Harrison (1983), who showed that the predictive reliability of mice for quantitative toxicity of five chemotherapeutic drugs in humans was at least as good, if not better, that the predictive reliability demonstrative by dogs and monkeys. More recently,

Newell *et al.* (2004) presented data on predictive performance of rats and mice to demonstrate qualitative human toxicity when given 39 novel cancer chemotherapeutic agents. They claim that nonrodent species are unnecessary for identification of a safe Phase 1 starting dose for human trials. Furthermore, the two rodent species (rats and mice) used gave similar quantitative and qualitative results.

The arrival of genetically engineered mice has further enhanced the relevance of using mice in research, especially in terms of basic mechanistic research and applied screening for genotoxicity and carcinogenicity. Transgenic mice carrying a human gene and expressing the protein are said to be "humanized." These animals are invaluable in drug assessment especially when the drug interacts only with the human protein (Bolon 2004). Zambrowicz and Sands (2003a) showed that the KO phenotype of mice correlated well with the molecular targets of the 100 best selling drugs available to the U.S. market. Zambrowicz *et al.* (2003b) compared the physiology of KO mice, where the deleted gene was known to produce a novel target for each of the 24 new drugs in the developmental pipeline of the 10 largest pharmaceutical companies, and found that 85% of these targets demonstrated a sound biologic rationale for the selected disease.

Transgenic and KO mice have been used successfully to screen new drug candidates for safety and to elucidate basic mechanisms of toxicity. A large number of drug metabolizing enzyme (DME) KO lines have been employed in safety screening. DMEs may be involved in the safe metabolism of a drug or they may generate toxic intermediates. Removal of the DME gene may result in the animal being more sensitive to the test article, and it may provide protection against toxic effects of the drug. For instance, KO mice lacking the *NQO-1* gene have increased menadione toxicity, and mice lacking the *CYP2E1* gene are resistant to acetaminophen hepatotoxicity (Henderson and Wolf 2003).

Murine models are also available for evaluation of chemical mutagenicity (Big Blue Mouse; Stratagene, La Jolla, CA and Muta Mouse; Covance Research Products, Denver, PA). These marker genes are present in mice of different genetic backgrounds (Bolon 2004). KO lines have been created with the increased sensitivity to chemically induced carcinogenesis. Mice carrying a single *p53* allele, or over expressing Ha-*ras*, or having a complete deletion of the *Xpa* (xeroderma pigmentosum) gene, have all been used for screening xenobiotics in vivo (Bolon 2004). These same transgenic and KO models are useful in screening environmental chemicals for toxicities (Jacobson-Kram *et al.* 2004).

It is clear that the mouse will continue to be essential for discovery and in evaluating the safety of new drugs. Equally relevant is the development of clinical chemistry assays needed to study the associated metabolic events in mice (especially in transgenic and KO lines) and assess serum/plasma biomarkers. These biomarkers should either be quantitative measures of the biologic effects (which provide informative links between mechanism of action and clinical effectiveness) or surrogate markers (which are quantitative measures that predict effectiveness). In this arena, the field of proteomics is likely to make a dramatic impact on clinical chemistry.

## II. METHODS OF ANALYSIS AND INSTRUMENTATION

### A. Introduction

Although we hope all readers of this chapter will benefit from this section on assays and instruments, the primary purpose of this chapter is to briefly introduce the reader to areas where methods in clinical chemistry are changing and provide sources of information for services, reagents (including test kits), and instrumentation, specifically for testing biomarkers (including traditional analytes) in mouse serum, plasma, or urine. Those seeking a detailed description of clinical chemistry methods and instruments should refer to *The Fundamentals of Clinical Chemistry* (Burtis and Ashwood 2001).

### B. The Changing Nature of Clinical Chemistry

Some dramatic changes have occurred over the past two decades that have revolutionized the discipline of clinical chemistry. Historically, most analytes were measured by a colorimetric end point assay (based on the binding of an analyte to another molecule creating a new substance that absorbs light of a specific wavelength). Some of these analytes and the newer biomarkers are now measured by enzymatic tests that have higher specificities and sensitivities. Another change has been the substitution of electrochemical assays, such as ion-selective electrodes, for flame photometry used in the quantitation of sodium and potassium. Perhaps the change that has had the largest impact on clinical chemistry is the development of monoclonal antibodies and their use as reagents in immunoassays (to be discussed later). Many commercial companies now offer services that measure analytes and biomarkers in the blood of mice using a combination of colorimetric-, enzymatic-, electrochemical-, and immunologic-based methods, and many of the instruments used are capable of running multiple assay types simultaneously.

The use of laboratories that offer validated assays specifically for mouse blood is important to ensure accurate and precise results. A summary of a few laboratories that provide validated murine assays is presented in Table 6-1.

### C. Immunologic Methods to Detect Mouse Analytes

The increasing availability of mouse-specific reagents has resulted in many new assay techniques that provide a high

TABLE 6-1
CLINICAL CHEMISTRY SERVICES AVAILABLE

| Name | Address | Phone | Contact |
|---|---|---|---|
| Analytics, Inc. | 200 Girard Street<br>Suite 200<br>Gaithersburg, MD 20877 | 301-921-0168<br>800-237-2815 | Saroj R. Das |
| Antech Diagnostics<br>GLP | 507 Airport Blvd.<br>Suite 113<br>Morrisville, NC 27560 | 800-872-1001<br>×4374<br>919-277-0822 | Doug Neptun<br>doug.neptun@antechmail.com |
| Charles River DDS, Clinical Chemistry Laboratory, Laboratory Animal Diagnostic Services | 57 Union Street<br>Worcester, MA 01608-1114 | 508-890-0100 | |
| Idexx Laboratories<br>Pre-clinical Research | 3 Centennial Drive<br>Suite 1<br>North Grafton, MA 01536 | 207-439-9291<br>800-551-0998 | Anita Perreault<br>anita-perreault@idexx.com |
| Laboratory Corp. of America<br>Preclinical | 1904 Alexander Drive<br>Research Triangle Park, NC 27709 | 800-533-0567<br>×4338 | Monica Hamrick<br>hamricm@labcorp.com |
| Toxikon Corp. | 15 Wiggins Ave<br>Bedford, MA 01730 | 781-275-3330<br>×166 | Steven Presti<br>steve.presti@toxikon.com |

degree of sensitivity and specificity. Growth in the number of reagents capable of quantitating analytes in mouse serum is illustrated in Table 6-2. Techniques employing nonisotopic labels for detection such as enzyme cascade, fluorescence, chemiluminescence, and electrochemiluminescence, are rapidly replacing older radioimmunoassay (RIA) techniques. Enzyme immunoassays, such as enzyme-linked immunosorbent assay (ELISA), enzyme-multiplier immunoassay technique (EMIT), and cloned enzyme donor immunoassay (CEDIA), provide quantitative results based on photometric methods. Enzyme immunoassay is popular because it generates compounds that can be quantitated photometrically. Typical enzymatic labels include β-galactosidase, horseradish peroxidase, alkaline phosphatase, and glucose-6-dehydrogenase. In addition these ELISA test kits are compact, easy to use, and quantitated with inexpensive instruments. These kits (usually in a well format) are available for quantitation of a wide range of mouse serum and plasma biomarkers (Table 6-3).

TABLE 6-2
NUMBER OF PRODUCTS AVAILABLE TO DETECT MOUSE SERUM ANALYTES[a]

| Products | 1994–1995 | 2004–2005[c] |
|---|---|---|
| Immunoassay kits[b] | 24 | 228 |
| Monoclonal antibodies | 32 | 4137 |
| Polyclonal antibodies | 56 | 3402 |

[a]As listed in *Linscott's Directory of Immunologic and Biologic Reagents*. These figures exclude antibodies directed to immunoglobulins or CD markers on cells.

[b]In 2005 there were 180 mouse-specific EIA, 25 mouse-specific RIA, and another 48 kits that were multispecies specific.

[c]These represent unique target molecules. A single entry in this column may have as many as 12 different manufacturers for the same product.

Fluoroimmunoassay (FIA), which utilizes a fluorescent molecule as an indicator label, was previously subject to problems associated with background fluorescence. Today this problem has been largely resolved by using chelates of lanthanide as a label. Modifications of FIA have eliminated the need to separate free from bound label (homogenous assay).

Chemiluminescent and electrochemilumiscent immunoassays are similar to enzyme immunoassays, except that quantitation of results is based on the emission of light after a chemical label is exposed to an oxidation reaction (chemiluminescence) or to an electrochemical reaction (electrochemiluminescence).

The number of biomarkers that can be quantified by commercially available test kits based on immunoassays is likely to grow at unparalleled rates as a result of proteomics research where mice are the favored model.

### D. Multiplex Technology

Mulitplex immunoassay is a unique technology that combines four distinct components in a manner that allows for simultaneous analysis of up to 90 different analytes from 50 μL of serum/plasma. The core component is an inexpensive, consumable, 5.6 μm diameter polystyrene microsphere that are encoded into 100 different fluorescent color sets using two fluorophores (red and infrared) at multiple concentrations. The second component is a biologic assay (combined with an

TABLE 6-3
COMMERCIALLY AVAILABLE ELISA KITS FOR QUANTITATION OF MOUSE ANALYTES

**1. Adhesion Molecules/Lectins**
SICAM-1
sE-Selectin (CD62E)
sL-Selectin (CD62L)
sP-Selectin (CD62P)
SVCAM (CD106)

**2. Autoimmunity Circulating Immunocomplexes**
Rheumatoid factor IgG
Rheumatoid factor IgM
Anti-cardiolipin Ab
Anti-collagen type 1 Ab
Anti-collagen type 2 Ab
Anti-ds-DNA Ab
Anti-ss-DNA Ab
Anti-histone Ab

**3. Chemokines and Receptors**
6ckine/CCL21
BLC/BCA/CXCL13
CCL28
CTACK/CCL27
* CXCL16
* CX3CL1
Eotaxin/CCL11
* Eotaxin-2/MPIF-2/CCL24
* I-TAC/CSCL11
IP-10/CRG-2/CXCL10
JE/MCP-1/CCL2
KC
* LIX
MCP-5/CCL12
* Lymphotactin
* Lungkine
* MDC/CCL22
MIF
MIG/CXCL9
MIP-1α/CCL3
MIP-1β/CCL4
MIP-1 γ/CCL9/10
MIP-2
* MIP-3α/CCL20
* MIP-3β
PF4
RANTES/CCL5
SDF-1α/CXCL12
TARC/CLL17
* TECK/CCL25
* TCA-3/CCL1

**4. Cyclic Nucleotides Prostaglandins, Thromboxanes, Leukotrienes, Lipoxygenase, Cyclic Nucleotides**
CAMP
CGMP
PGE-1
PGE-2
PGD-2
8-Isoprostane
6-Keto-$PGF_1\alpha$
$PGF_2\alpha$
11β-$PGF_2\alpha$
13,14-dihydro-15Keto-$PGF_2\alpha$
15dihydro-$PGJ_2$
Urinary prostacyclin
$TXB_2$
2,3-dinor $TXB_2$
11-dehydro $TXB_2$
$LTB_4$
$LTC_4$
$LTE_4$
Cysteinyl-leukotriene
12(S)HETE
Lipoxygenase Inhibitor Screening Assay

**5. Growth Factors (*Paired Ab)**
IGF-1
IGF-2
* IGF-11
* IGF-BP-2
* IGF-BP-3
* IGF-BP-6
G-CSF
GM-CSM
M-CSF
Placental growth factor 2
Stem cell factor
PDGF-BP
PDGF
* FGF-basic
VEGF
* sVEGF R1/Flt-1
* sVEGF R2/Flk-1
* VEGF-D
EGF

**6. Hormones and Neurotransmitters**
ACTH
Corticosterone
Cortisol
DHEA
Endothelin-1
Endothelin-3
Estriol
Estradiol-17β
Insulin
Leptin
Adiponectin
Growth hormone
Erythropoietin
Oxytocin
Progesterone
Substance P
Testosterone
$Arg^8$-vasopressin
Parathyroid hormone
Insulin-like growth factor-1 (IGF-1)
Resistin
CGRP
Aldosterone
1111-Keto testosterone
Histamine
Osteocalcin
Thrombopoietin
Obestatin

*Matched antibodies available.

Continued

TABLE 6-3
COMMERCIALLY AVAILABLE ELISA KITS FOR QUANTITATION OF MOUSE ANALYTES—CONT'D

| |
|---|
| **7. Immunoglobulins** |
| IgA |
| IgE |
| IgG total |
| IgGFc |
| IgG1 |
| IgG2a |
| IgG2b |
| IgG3 |
| IgM |
| **8. Interleukins and Interferons** |
| IL-IRAntagonist |
| IL-1α |
| IL-1β |
| IL-1R1 |
| IL-2 |
| IL-3 |
| IL-4 |
| IL-5 |
| IL-6 |
| KC |
| IL-7 |
| IL-10 |
| IL-11 |
| IL-12 |
| IL-12p40 |
| IL-12p70 |
| IL-13 |
| IL-17 |
| IL-18 |
| IFN-α |
| IFN- γ |
| **9. Oxidative Injury** |
| STAT-8-isoprostane |
| iPF2α-VI |
| 8-Isoprostane |
| Glutathione |
| Glutathione peroxidase |
| Glutathione reductase |
| Lipid hydroxyperoxide |
| Hydrogen peroxide |
| Superoxide dismutase |
| Catalase |
| Protein carbonyl |
| NO-nitrate/nitrite colorimetric |
| NOS assay |
| **10. Proteases and Inhibitors** |
| MMP-3 (matrix metalloproteinases) |
| Pro-MMP-9 |
| MMP-9 |
| Tissue inhibitor of metalloprotease 1 (TIMP-1) |
| **11. TGF-β and Tumor Necroses Factor Superfamilies and Receptors** |
| TGF-β |
| BMP |
| TNF- α |
| TNFRI |
| TNFR11 |
| TNFSF11(TRANCE) |
| Ghrelin |
| Fas ligand |
| gFas(TNFRSF6/CD95) |
| Osteoprotegerin/TNFRSF11B |
| * TROY/TNFRSF9 |
| * CD40 (TNFRSF5) |
| * CD30 (TNFRSF8) |
| * CD30L |
| **12. Other Proteins** |
| Agouti-related protein |
| Albumin |
| ANP |
| C3a |
| Endostatin |
| Flt-3 ligand |
| Ileal fatty acid binding protein |
| Lipopolysaccharide binding protein |
| LIF |
| Mannose binding lectin A |
| Mannose binding lectin B |
| Microalbumin |
| Myoglobin |
| Orexin A |
| Orexin B |
| Osteopontin |
| p38 alpha p53 |
| Serum amyloid A |
| Tau |

orange fluorescent reporter molecule) that is built onto the surface of the microspheres. A diverse range of biologic assays can be built onto the microsphere surface, including immunoassays, nucleic acid assays, enzymatic reaction assays, or receptor-ligand analysis assays. The third component is a flow cytometer that focuses the microspheres into a single file in front of a two interrogating lasers, which allow for high throughout. One laser is a red diode emitting at 635 nm, which illuminates each microsphere. The resulting red and infrared fluorescence provides classification information for that particular microsphere set. The other laser is a green YAG diode emitting at 532 nm that excites the orange fluorescent reporter molecules of the surface of the microsphere, providing a quantitative signal for that particular biologic assay. The last component is digital signal processing data acquisition hardware that provides the speed necessary to read the microspheres at up to 5000 per second.

Because multiplex technology can analyze a wide range of biomarkers simultaneously, dynamic reference results (plasma profiles) can be developed based on the changing concentrations of the biomarkers during the course of a disease. The known and potential applications of developing plasma profiles of diseases are powerful. Plasma profiles can be used to screen and identify diseases (especially during the early development

of a disease) and characterize the efficacy of drugs targeted against these diseases. Multiplex technology can help elucidate new biomarkers of disease. Furthermore, plasma profiles could also potentially be developed to monitor the blood concentrations of drugs as well.

### E. Sources of Information

Investigators seeking additional information on availability of reagents and test kits should refer to "Clinical Laboratory Reference" (Nelson 2006) and the *Linscott's Directory of Immunologic and Biologic Reagents* (Linscott 2005). Sections of these two references provide the names and addresses of sources. The catalogs of individual companies may be fruitful, but many reagents for use in human blood and have not been validated for use in other species (including the mouse).

Each of the various instruments mentioned in this section may be found in the *8th Annual Analyzers Buyer's Guide* (Advance for Administrator of the Laboratory 2003). This guide lists all manufacturers and gives detailed information on each instrument including technology platforms, methods used, through-put capability, purchase price, maintenance costs, reagent package cost, as well as a variety of options available. Contact information, including Web site, is available for each manufacturer.

Additional information regarding clinical chemistry on animal blood is available on the Web site of the Division of Animal Clinical Chemistry at the American Association of Clinical Chemistry (www.aacc.org/divisions/animal).

## III. SAMPLING

With a mean body size of 35 g, adult mice have approximately 2.4 ml of blood volume, allowing 75 μl to be removed weekly without consequence to health and welfare (Loeb 1997; Loeb and Quimby 1999). Four blood collection sites for acquiring 75 μl of blood repetitively from adult mice include the orbital plexus, the tail vein, the jugular vein, and cardiac puncture (Quimby 1999b). General anesthesia is recommended for orbital plexus and cardiac puncture, although Terril-Robb *et al.* (1996) claim that topical application of proparacaine hydrochloride is an acceptable procedure for collecting orbital plexus blood. The jugular vein is punctured using a lancet directed at the rear of the jaw, exposing the jugular vein and its tributaries and the submandibular and facial veins (Golde *et al.* 2005). Collection of blood from any of these veins at this site works well, and the animal does not require anesthesia or a restraint device. The blood should be collected with a small centrifuge tube or capillary tube. Nerenberg and Zedler (1975) describe a vacuum apparatus used to collect larger volumes of blood from the tail vein. Lewis *et al.* (1976) found that heparinization of mice before tail bleeding increases the yield. Prewarming the mouse under a lamp or through immersion in warm water facilitates tail bleeding. The use of heparinized microhematocrit tubes, during tail or orbital plexus bleeding can maximize the plasma volume with minimal hemolysis (Quimby 1999b). Hem and Smith (1998) recommend a saphenous vein prick method over the orbital plexus method for collecting repeated small volumes from conscious mice. MacLeod and Shapiro (1988) used indwelling right atrial catheters for repetitive bleeding of conscious, unstressed mice. Disadvantages of specific procedures have been described. Sakaki *et al.* (1961) reported sympathetic nervous system stimulation associated with tail bleeding and the mixing of venous and arterial blood with the orbital plexus method. Patrick *et al.* (1983) found that, compared to jugular vein collection, cardiac puncture was associated with higher plasma glucose concentrations and lower creatine kinase (CK) activity. Plasma glucose concentrations are higher in blood collected from the orbital sinus compared to the tail vein.

Differences associated with the method of anesthesia are also important. Halothane, methoxyflurane, isoflurane, and pentobarbital sodium have been widely used and are considered safe. However, caution should be exercised in the interpretation of certain chemistry values. Higher plasma glucose levels are reported with tail vein sampling in mice anesthetized with pentobarbital or methoxyflurane compared with conscious mice (Chuang and Luo 1997). Plasma glucose levels are higher after collection from the orbital plexus if pentobarbital or proparacaine hydrochloride is administered. Methoxyflurane has no glucose elevating effect on samples collected from the orbital plexus. Cunliffe-Beamer (1983) claims that carbon dioxide narcosis provides sedation and analgesic for 1–2 minutes and is an appropriate anesthetic for orbital plexus bleeding.

When greater volumes of blood are required, four collection sites can be used that require anesthesia and subsequent euthanasia of the mouse. These sites include the jugular vein (Ambrus *et al.* 1951), the abdominal aorta (Lushbough and Moline 1961), the brachial artery (Young and Chambers 1973), and the heart (Cubitt and Barrett 1978; Mitruka and Rawnsley 1977).

Blood collection from a newborn mouse can be accomplished by decapitation. Injection of two units of heparin subcutaneously several minutes before decapitation may allow collection of up to 40 μl of blood (20 μl of plasma).

Although collection of urine is possible in mice, it is impractical due to anatomic and physiologic restrictions. The total urinary bladder volume is less than 0.5 ml, and total urinary output per day is less than 2 ml (Jung *et al.* 2003), meaning that mice frequently micturate. This restricts the amount urine that can be collected at any one time point and, therefore, will also restrict the number of analytes that can be assessed from any one urine sample.

To be able to collect enough urine for assessment from a single live mouse, mice can be placed in a plastic cage without absorbent bedding material and urine then collected from the bottom of the cage after 4–6 h. However, a disadvantage of this technique is that the urine is exposed to the contamination by feces and other environmental contaminants and organisms. Pooled urine samples collected from a group of live mice using the same technique can increase the total amount of urine collected but will not allow specific results to be related back to specific individuals from that group. Mice removed quickly from their cage and held over a piece of parafilm will frequently void and the parafilm helps prevent the urine from spreading. Urine can also be collected directly from the urinary bladder from mice after euthanasia. This technique allows for a sterile sampling, but the volume that can be collected is restricted by total urinary bladder volume (0.5 ml). Often, the volume is much less because mice typically void urine from the urinary bladder at the time of death.

## IV. REFERENCE RANGES

### A. Introduction

Reference ranges refer to the range of an analyte or biomarker in a population that has not been selected for the presence of disease or abnormality. Reference ranges are usually generated from a large number of individuals from a population, so reference ranges for the same analyte or biomarker can vary between two different populations. Table 6-4 lists the reference ranges for selected analytes in two inbred strains and an outbred stock.

In some situations, reference ranges that have been published or generated by laboratories cannot be relied on to accurately determine the presence or absence of disease or abnormality. These situations include evaluating analytes and biomarkers in transgenic and KO mouse populations (especially in experiments in which population sizes are small) and evaluating certain analytes and biomarkers (such as those assessing immune function). Instead, baseline data compiled from controls (the population of wild-type mice from which the transgenic and KO mice were derived from) should be used.

### B. Variability

The usefulness of compiled baseline data depends on controlling a large number of variables known to influence chemistry determinations. Several studies have demonstrated significant differences in selected serum analyte concentrations with age difference in the same strain, between sexes in the same strain, and among strains (Everett and Harrison 1983). Some variables known to adversely affect the host include environmental factors, pathogens, and shipment. In addition, nutrition, time of sample collection, and storage techniques may all contribute to variability.

Age has an effect on analytes in the mouse. In an early study, Barrett *et al.* (1975) found that serum calcium levels in 4-month-old C3H/Fg and A/Fg inbred mice were significant higher than 7-month-old mice of the same strains. More recent and more comprehensive analyses completed by Loeb *et al.* (1996) reinforces the effect that age has on clinical chemistry parameters in five inbred strains and two F1 hybrids, including serum protein.

The effect sex has on chemistry parameters is most demonstrable with sex hormones, including follicle-stimulating hormone (FSH), luteinizing hormone (LH), and progesterone. Table 6-3 illustrates these differences between male and female mice, and between female mice in estrus and out of estrus.

Strain-associated changes appearing in healthy animals have been documented for complement components (Goldman and Goldman 1976), cholesterol (Dunnington *et al.* 1981; Meade and Gore 1982), testosterone (Ivanyi *et al.* 1972), cortisol-binding protein (Goldman *et al.* 1977), and serum protein (Borovkov and Svirdov 1975; Loeb 1997).

Cyclic biorhythms, whether circadian or ultradian, influence blood levels of various analytes in mice. Blood levels of adrenocorticotropic hormone (ACTH), corticosterone, growth hormone (GH), and LH may peak one or more times daily, and thus special attention must be given to both the time of collection and the order of sampling individuals between groups if between-group comparisons are to be made (Loeb 1997).

The degree of hydration, exposure to noise, degree of confinement, and environmental temperatures has all been shown to affect serum chemistry analytes (Quimby 1999b).

Diet is known to influence the blood levels of many analytes. Perhaps the best studied is the effect of atherogenic diets on serum cholesterol. Similarly, significant differences in both serum cholesterol and urea nitrogen are seen in mice maintained on a semipurified (AIN-76) diet. The mouse is unique among mammals because murine muscles do not contain carnosine or anserine. Carnosine serves as a source of histidine when histidine is restricted. Unlike other mammals, mice on histidine-free diets show signs of histidine deficiency. Fasting may also affect the levels of certain analytes (Quimby 1999b).

The presence or absence (axenic mice) of intestinal microbial flora is associated with dramatic changes in immunoglobulin (Ig) levels and may be associated with changes in other analytes as well. For example, axenic mice have significantly lower levels of IgA compared to conventional mice (Moreau *et al.* 1982). The presence of pathogens may be associated with dramatic changes in various analytes, even with subclinical infection. For instance, infection with lactic dehydrogenase-elevating virus (LDV) is associated with major elevations in serum lactic dehydrogenase, isocitric dehydrogenase, malic dehydrogenase, aspartate aminotransferase, and glutathione reductase activities (Quimby 1999b).

**TABLE 6-4**
CLINICAL CHEMISTRY REFERENCE RANGES FOR ADULT MICE[a]

| Analyte | Units | CD-1 | | C57BL/6 | | BALB/cBy | |
|---|---|---|---|---|---|---|---|
| | | M | F | M | F | M | F |
| Serum | | | | | | | |
| Glucose | mg/dl | 112 ± 38.1 | 97 ± 39.9 | 121.7 ± 33.2 | 134.4 ± 20.3 | 171.6 ± 57.2 | 174.9 ± 31.0 |
| Urea nitrogen | mg/dl | 38 ± 20.1 | 37 ±16 | 32.7 ± 3.5 | 23.6 ± 5.3 | | |
| Creatinine | mEq/l | 1.10 ± 1.45 | | 0.50 ± 0.08 | 0.84 ± 0.298 | 0.43 ± 0.14 | 0.45 ± 0.07 |
| Sodium | mEq/l | 166 ± 8.6 | 166 ± 4.1 | 166.7 ± 8.9 | 160.8 ± 4.40 | 157.8 ± 5.7 | 157 ± 6.70 |
| Potassium | mEq/l | 8.0 ± 0.85 | 7.8 ± 0.75 | | | | |
| Chloride | mg/dl | 125 ± 7.2 | 130 ± 3.9 | | | | |
| Calcium | mg/dl | 8.90 ± 2.06 | 10.30 ± 1.58 | | | 8.10 ± 0.80 | |
| Phosphorus | mg/dl | 8.30 ± 1.46 | 8.00 ± 1.85 | | | 5.95 ± 0.63 | |
| Magnesium | mg/dl | 3.11 ± 0.37 | 1.38 ± 0.28 | | | | |
| Iron | μg/dl | 474 ± 44 | 473 ± 16 | | | | |
| Alanine aminotransferase | IU/l | 99 ± 86.3 | 49 ± 22.6 | 41.4 ± 16.4 | 29.3 ± 7.1 | | |
| Aspartate aminotransferase | IU/l | 196 ± 132.6 | 128 ± 60.6 | 99.5 ± 33.4 | 73.6 ± 15.3 | | |
| Alkaline phosphatase | IU/l | 39 ± 25.7 | 51 ± 27.3 | 59 ± 11.4 | 118 ± 15.9 | | |
| Lactate dehydrogenase | IU/l | | | | | 378 ± 269 | |
| Protein, total | g/l | 44 ± 11.0 | 48 ± 8.5 | 53.9 ± 7.5 | 63.5 ± 8.8 | 55.7 ± 8.9 | 54.6 ± 8.3 |
| Albumin | g/l | | | 36.7 ± 5.2 | 46.4 ± 7.0 | 31.7 ± 4.7 | 39.3 ± 5.4 |
| Cholesterol | mg/dl | 114 ± 56.3 | 72 ± 20.1 | 94.8 ± 16.9 | 92 ± 15.9 | 150.4 ± 29.9 | 118.2 ± 36.1 |
| Triglycerides | mg/dl | 91 ± 58.5 | 53 ± 23.6 | 97 ± 21.1 | 78 ± 12.2 | | |
| Bilirubin | mg/dl | 0.4 ± 0.2 | 0.5 ± 0.35 | | | 0.7 ± 0.15 | |

| Analyte | Units | Male | Female | Female |
|---|---|---|---|---|
| Luteinizing hormone | ng/ml | 10–40 | 20–40 (basal) | 1500–2000 (proestrus) |
| Follicle-stimulating hormone | ng/ml | | 80–120 (basal) | 250–300 (proestrus) |
| Prolactin | ng/ml | <1 | 10–20 | |
| Growth hormone | ng/ml | | 1–90 | |
| Thyroid-stimulating hormone | ng/ml | | 300 | |
| Thyroxine | μg/dl | 7.4 ± 0.5 (BALB/c) | | |
| Corticosterone | μg/dl | 9 (start of dark period) 5 (start of light period) | 40 (middle of dark period) | |
| Epinephrine | pg/dl | 0–200 | | |
| Norepinephrine | pg/dl | 30–300 | | |
| Progesterone | ng/ml | | 5 (early proestrus) | 35 (late proestrus, estrus) |
| Estradiol | pg/ml | | 1–5 (basal) | |
| Testosterone | ng/ml | 1.5–2.0 | | |
| Urine | | | | |
| Volume | ml/16 h | 1.6 ± 0.9 | 1.7 ± 1.1 | |
| Specific gravity | | 1.0341 ± 0.005 | | |
| pH | | 5.011 | | |
| Osmolality | Osm/kg | 1.06–2.63 | | |
| Creatinine | mg/100g/24 h | 2.6 ± 0.91 | | |
| Glucose | mg/24 h | 0.53 ± 0.19 | | |
| Protein | mg/24 hr | 0.7 ± 0.33 | 3.2 ± 1.05 (B6C3F1) | |
| Albumin | mg/ml | 11.9 ± 0.2 | | |

[a]Summarized from Loeb and Quimby (1999).

Alterations in clinicopathologic parameters can be attributed to stress in mice. Landi *et al.* (1982) found that plasma corticosterone concentrations in mice tested within 48 h after arrival (by plane or truck transport) were significantly higher than tested after 48 h post arrival. Mice sensitized on arrival with sheep red cells as an antigen had significantly lower antibody titers, fewer plaque-forming cells, and a decreased delayed type of hypersensitivity reaction when compared to normal mice allowed to acclimate to the facility for 48 h before being sensitized. Elevated serum corticosterone levels can arise from excessive handling of mice prior to blood collection.

The effect of sample storage at various temperatures and for varying periods has been described. Falk *et al.* (1981) evaluated the effect of storage time (after freezing) on 20 serum analytes in six laboratory species. They found that in the mouse, only creatine kinase activity changed significantly with storage up to 28 days.

Hemolysis is a common problem during blood collection in mice. Hemolyzed samples are associated with changes in various enzymes, such as increased CK activity and decreased lipase activity. To minimize this, Everett and Harrison (1983) recommend heparinized plasma and careful selection of collection sites for routine chemical determinations.

The effect of lipemia, various anticoagulants, and pharmacologic agents on clinical chemistry values has been reviewed for domestic animals (Meyer and Harvey 1998).

### C. Quality Assurance

The issue of quality control and test validity has been thoroughly discussed for common domestic animals and is applicable to chemistry determinations in mice (Meyer and Harvey 1998). Quality assurance in clinical chemistry determinations is important to ensure that consistently accurate and precise results are achieved. Everett and Harrison (1983) stressed the importance of a clinical pathology quality assurance program that includes regular assays of pooled and commercially prepared pre-assayed sera. In addition, they encourage participation in a subscription quality assurance program, such as with a veterinary laboratory association. The within-day and day-to-day coefficient of variation should be known for each analyte measured.

Due to the tremendous number of variables known to influence clinical chemistry values in mice, it is often prudent to test adequate numbers of control specimens along with the experimental samples. This technique is often impractical when tests are being conducted strictly for diagnostic purposes, and in those situations, compiled values that are controlled for as many variables as possible may be sufficient.

### D. Statistics

The values that define a reference range for a particular analyte or biomarker in normal or healthy mice may be described using several methods. Regardless of the distribution of data, it is generally useful to describe the limits that include 95% of the test results in a disease-free population. For values exhibiting a Gaussian distribution, parametric methods (such as mean and standard deviation) are appropriate. For Gaussian distributed data, this is the range that includes two standard deviations above and below the mean.

Certain murine analytes have non-Gaussian distributions and must be evaluated using nonparametric methods. A variety of methods are available, including the percentile method and logarithm-transformed data analyzed with parametric methods. The method of percentile estimates is more vulnerable to bias due to extreme values (outliers) than is the log-transformed parametric method. Boyd (1985) asserts that a sample size of at least 120 is required to give 90% confidence intervals using the percentile method, whereas a sample size of 50 may give reliable ranges if parametric analyses are used. Neither statistical method just described will replace raw data in certain situations, such as when assessing analytes for immune function, nor when using data derived from wild-type mice as comparison baseline data for transgenic and KO mice.

## V. SPECIFIC TESTS

### A. Introduction

This section is intended to serve as a review of specific tests for routine analytes. Additionally, a more comprehensive discussion is presented for analytes and biomarkers involved in two areas of translational research of intense interest (obesity/diabetes and atherosclerosis) and novel biomarkers of disease (such as immune function tests). Only analytes and biomarkers for which there are currently available commercial tests for mice serum, plasma, and/or urine are discussed; certain analytes and biomarkers for which commercially available tests are not available for the mouse (such as calcitonin) are not covered. For more complete information for the routine analytes, please refer to Loeb and Quimby (1999).

### B. Glucose and Carbohydrate Metabolism

#### 1. Glucose and Its Measurement

Glucose is the main source of energy in mice. Blood glucose concentrations depend on the rates of entry and the removal rate from the blood. The rate of entry is dependent on intestinal absorption of dietary sources of glucose, the breakdown of body glycogen stores (glycogenolysis), and synthesis from gluconeogenic metabolites (gluconeogenesis). The removal rate is mainly dependent on insulin, which is released from β-cells of the pancreatic islets. Insulin promotes cellular uptake of blood

glucose (mainly in muscle, liver, and fat) by stimulating the translocation of glucose transporters, GLUT-1 to GLUT-7 to the cell membrane. When removed from circulation, blood glucose may either be utilized (to maintain cell function) or converted to fat and glycogen in liver and muscle as an energy store. However, the effect of insulin is modulated by other hormones (such as glucagon, corticosterone, GH, epinephrine, somatostatin, and amylin) that ultimately result in the tight control the levels of blood glucose, depending on tissue demands for energy. Glucagon is released from α-cells of the pancreatic islets in response to low circulating glucose, stimulating the liver to increase circulating glucose through glycogenolysis and gluconeogenesis. Corticosterone and GH antagonize the action of insulin. Epinephrine suppresses insulin release and stimulates glucagon release and glycogenolysis. Somatostatin suppresses both insulin and glucagon secretion, whereas amylin increases blood glucose, blood insulin, and insulin resistance (Burtis and Ashwood 2001; Kaneko 1999).

Glucose determinations are generally conducted on fresh serum. However, plasma glucose determination is acceptable if delay of greater than 30 minutes before separation of erythrocytes is anticipated. In this case, fluoride should be used as the anticoagulant because it inhibits glycolysis by erythrocytes. Blood glucose in mice is measured using the hexokinase or glucose oxidase. These tests may be performed as an analytical method or by using glucose oxidase coated test strips (for urine glucose), or small portable analyzers (Seidelmann *et al.* 2005). Assessment of long-term average blood glucose levels in mice is also available by RIAs measuring glycosylated hemoglobin and glycosylated serum proteins (collectively known as fructosamines) (Gould *et al.* 1986).

The Mouse Phenome Database lists serum glucose levels of 41 strains with blood collected after a 4-h fast on 7–9 week old males and females on a standard laboratory diet. For all mice, the overall mean was 179 ± 30.9 mg/dl (Naggert *et al.* 2003), but blood glucose levels varied with age, sex, and strain. Females of a strain tended to have lower levels than males, with LP/J mice showing the lowest values (F = 125 ± 22.0 and M = 146 ± 18.8 mg/dl) and C57Bl/10J mice showing the highest values (F = 230 ± 25.5; M = 263 ± 57.3 mg/dl). In mice serum glucose levels decrease between the 3rd and 12th month of age and in C57BL/6 and BALB/c strains the glucose level rises again after 24 months (Loeb 1997).

Serum or plasma glucose levels may also vary depending on the site of collection and anesthetic used (Quimby 1999b). Patrick *et al.* (1983) found that compared to jugular vein collection, cardiac puncture was associated with higher blood glucose. Differences associated with the method of anesthesia are also important. Higher plasma glucose levels are reported after tail vein sampling under either pentobarbital or methoxyflurane compared to sampling conscious mice (Chuang and Luo 1997). Plasma glucose levels were higher after orbital plexus sampling of mice if pentobarbital or proparacaine hydrochloride was provided. Methoxyflurane had no glucose elevating effect on samples collected from the orbital plexus; however, in conscious animals, plasma glucose levels were higher in blood collected by retro-orbital sinus compared to tail bleeding.

Hyperglycemia (increased blood glucose levels) in the mouse can be due to increased peripheral resistance of tissues to insulin (such as with exogenous corticosteroid administration, increased endogenous corticosterone release, glucagons administration, and GH administration), and diabetes (see discussion later). Hypoglycemia (decreased blood glucose levels) can be due to excessive circulating insulin (such as with excessive insulin administration and transgenic mice with β-cell tumors of the pancreatic islets), reduced glycogen stores (such as with advanced liver disease), excessive glucose use (such as with pregnancy, septicemia, and neoplasia), and reduced glucose intake (such as with starvation and diseases of malabsorption).

### 2. Diabetes

Diabetes in mice is defined as persistent blood glucose levels of greater than 300 mg/dl in fasting mice. This level also corresponds to the renal threshold for glucose urine excretion in mice, so urine glucose assessment is useful in this regard for mice. Nonobese diabetic mice (a model of type 1 diabetes) have nonfasting plasma glucose levels in young prediabetic mice between 130–180 mg/dl, which rises to >300 mg/dl between 10–30 weeks of age (Leiter 1997). Genetically modified mice have been identified for virtually every ligand and receptor regulating glucose metabolism and, depending on the gene mutation, may exhibit altered levels of plasma glucose. For example, adenosine monophosphate-activated protein kinase (AMPK) is a critical enzyme in energy metabolism (including cellular glucose uptake and fatty acid oxidation in muscle, and fatty acid synthesis and gluconeogenesis in hepatocytes). Using KO mice, Shaw *et al.* (2005) demonstrated that kinase LKB1 is an important activator of AMPK in the liver under energy-stress conditions, and that KO mice deficient in kinase LKB1 were persistently hyperglycemic. The authors also demonstrated that kinase LKB1 is the target of the type 2 diabetic therapeutic drug, metformin.

Glucose tolerance tests (GTT) have been performed in mice. One-, 3-, and 4-h tests have been conducted. In the 1-h test, glucose concentrations are evaluated before and 1 h following the administration of 2 mg/g glucose administered intraperitoneally (IP) (Oldstone *et al.* 1984). The 3-h test compares pre-injection serum to serum collected at 15, 30, 60, and 120 minutes following administration of 3 mg/g glucose given IP (Hotamisligil *et al.* 1996). A sensitive 4-h GTT has also been described in which mice are given 10 ml/kg of 10% glucose orally (Gates *et al.* 1972). The hypoglycemic response to insulin in mice has also been described (Hotamisligil *et al.* 1996). Kaku *et al.* (1988) measured glucose, glycosylated hemoglobin and insulin levels in six inbred strains undergoing GTT (either in fed or fasted mice). To estimate the number of genes involved in phenotypic

differences in glucose tolerance, the least glucose tolerant strain (C57BL/6) was bred to the most tolerant strain (C3H/HeJ) and F1 hybrids and backcross animals tested. The authors concluded that glucose tolerance in six commonly used inbred strains is a polygenic trait.

### 3. Relationships Between Obesity and Diabetes

Thrifty genes have been hypothesized to give early human hunter-gatherers a survival advantage by providing an economic mechanism to store energy during periods of famine (Zimmet and Thomas 2003). Because the most efficient mechanism for energy storage is through promotion of adipose tissue, it seems reasonable that at least some of these "thrifty" genes may function in this manner. Another hypothesis holds that during periods of famine it is essential to conserve glucose for use by the brain and that the mechanism responsible involves insulin resistance in peripheral tissues (Neel 1962). Should both hypotheses be true it is easy to envision an association between obesity and type 2 diabetes, especially where sedentary lifestyle and unrestricted access to food occur together (Lazar 2005).

One candidate thrifty gene encodes the hormone leptin. Leptin is produced by adipose tissue, and its absence leads to obesity and insulin resistance. During times of high adipose storage blood levels of leptin are high and it promotes energy metabolism and inhibits food intake. The opposite occurs during starvation. But leptin is only one of a number of adipokines, secreted by adipose tissue, which aid in the regulating appetite and metabolism. In fact the location of the adipose tissue, the size of average adipocytes, and adipocyte metabolism of glucose and corticosteroids each modify the endocrine function of adipose tissue (Lazar 2005). Among the proteins secreted by adipose tissue are adiponectin, adipsin, resistin, visfatin, tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), macrophage chemoattractant protein-1 (MCP-1), plasminogen activator inhibitor-1 (PAI-1), angiotensinogen, SAA, and α-acid glycoprotein. Like adipocyte-derived free fatty acids, which have been shown to contribute to insulin resistance in liver and muscle, most of these proteins are capable of modulating glucose metabolism and insulin action.

Adiponectin and visfatin work synergistically with insulin to enhance glucose uptake by muscle and block glucose synthesis by the liver (Hug and Lodish 2005). Blood levels of visfatin increase in obesity and the cytokine can bind to and stimulate the insulin receptor (Fukuhara *et al.* 2005). Blood levels of adiponectin negatively correlate with body mass and are lower in obese humans and mice, suggesting that reduced mRNA expression of the adiponectin gene may be involved with obesity (Masaki *et al.* 2004). Increases in adiponectin downregulate the hepatic expression of TNF-α. It mediates its antidiabetogenic effects via receptors on peripheral tissues, especially liver.

TNF-α, resistin, and IL-6 each induce resistance to insulin. However TNF-α also suppresses expression of adipocyte specific genes; resistin maintains glucose during fasting; and IL-6 production increases in the obese. The cytokines TNF-α and IL-6 are proinflammatory, are also produced by monocytes, and act on the liver to produce acute phase reactants. They also induce suppressor of cytokine signaling-3 (SOCS-3), an intracellular signaling molecule that impairs neuronal signaling by leptin and insulin, and thus causes resistance to the central actions of both hormones (Schwartz and Porte 2005). Resistin mediates its effects principally by decreasing the expression of gluconeogenic enzymes in the liver (Banerjee *et al.* 2004).

Certain cytokines, increased endoplasmic reticulum stress, chronic hyperglycemia, chronic hyperlipidemia, and oxidative stress may all induce apoptosis of insulin producing β-cells in the islets of the pancreas (Rhodes 2005). Insulin receptor substrate-2 (IRS-2) is a key molecule promoting β-cell growth and survival. These molecules act immediately downstream from surface receptors for insulin and insulin-like growth factor-1 and inhibition of IRS-2 has been shown to lead to insulin resistance. Inhibition may result from accumulation of reactive oxygen species in β-cells chronically exposed to increased glucose metabolism or from chronic exposure to elevated fatty acid (which through production of long chain acyl-CoA active protein kinase C-isoforms degrade IRS-2). Leptin has been shown to modulate interleukin-1β (IL-1β), a potent inducer of apoptosis. TNF-α and IL-6 induce β-cell apoptosis by activating the transcription factor nuclear factor κβ (NF-κβ).

Centrally the actions of both insulin and leptin take place in the mediobasal hypothalamus, where the neurons exert potent effects on food intake and energy expenditure. Here neurons co-express neuropeptide Y (NPY) and Agouti-related peptide (AgRP), which stimulate food intake and reduce energy expenditure. Leptin and insulin inhibit these neurons. Under conditions of reduced leptin and insulin signaling, NPY increases, inducing hyperphagia, weight gain, insulin resistance, and glucose intolerance. The anabolic effects of AgRP arise from its antagonism of the melanocortin receptors MC3r and MC4r, which serve to limit food intake. Blockage of MC3r and MC4r leads to weight gain and insulin resistance. Precursor proopiomelanocortin (POMC) and POMC neurons in the arcuate nucleus are stimulated by leptin and insulin and the resultant production of melanocortin and its binding to MC3r and MC4r inhibits food intake and promotes weight loss (Schwartz and Porte 2005). However, in the absence of leptin, as seen in $Lep^{ob/ob}$ mice, neurons of the arcuate nucleus of the hypothalamus are permanently disrupted and treatment in adulthood cannot reverse this defect (Bouret *et al.* 2004). Orexin A (hypocretin-1) and orexin B (hypocretin-2) are neuropeptides produced in the lateral hypothalamus by neurons with axonal projections to many sites including those that control feeding behavior and sleep/wakefulness (Taylor and Samson 2003). Orexin KO mice develop hypophagia with obesity and insulin-resistant diabetes (Hara *et al.* 2001). Ghrelin, a peptide made predominantly by the stomach, is also known to act

centrally and affect food intake and increase secretion of GH (Ghigo *et al.* 2004; Korbonits *et al.* 2004). In the periphery leptin has been shown to specifically repress RNA levels and enzymatic activity of hepatic stearoyl-CoA desaturase-1 (SLD-1), which catalyzes the biosynthesis of monounsaturated fatty acids. This effect was found to be an important metabolic action of leptin (Cohen *et al.* 2002). Leptin resistance, a common feature of obesity in mice and humans, has also been shown to result, in part, from the shedding of membrane-bound hepatic leptin receptors into the plasma, where soluble receptors modulate circulating leptin levels and possibly its biologic activity (Cohen *et al.* 2005). Thus the connections between factors regulating obesity and insulin resistance (diabetes) are complex and occur both centrally and peripherally. Further investigations in mice will involve quantifying glucose, adipokines, insulin, leptin, soluble leptin receptor, and SLD-1. For further discussion of mouse models of obesity and diabetes please refer to Chapter 19 in this text.

#### 4. Measurement of Hormones Associated with Carbohydrate Metabolism and Food Intake

Insulin, leptin, amylin (also called islet amyloid polypeptide, IAPP), glucagon, ghrelin, obestatin, orexin A, orexin B, GH, and corticosterone have been measured in mice, and RIA and ELISA kits are commercially available (see Table 6-3). In addition, these hormones may be measured as part of a multiplex panel (see "Multiplex Technology" section). Leptin values in C57BL/6, 129, and FVB/N strains range from 1–3 ng/ml, insulin values range from 2.2–5.2 μ IU/ml, corticosterone levels range from 5–40 μg/dl, and GH ranges from 1–90 μg/ml. Epinephrine is measured by RIA, and the range for mice is 0–200 pg/dl (DePaolo and Masoro 1989).

#### 5. Measurement of Adipokines

Adipokines secreted entirely by adipose tissue include adiponectin, adipsin, and resistin. Adiponectin and resistin have been measured in mouse serum and commercial ELISA test kits are available for this species. A polyclonal antibody that cross-reacts with a conserved sequence of mouse adipsin has been created (Searfoss *et al.* 2003). TNF-α, IL-6, and visfatin are adipokines that are synthesized also by macrophages and lymphocytes; as such they provide a common link between regulation of obesity, resistance to insulin and inflammation (Lazar 2005). Both TNF-α and IL-6 may be measured by commercially available ELISA kits (see the "Cytokines and Chemokines" section). A method has also been published for measurement of visfatin (Fukuhara *et al.* 2005).

#### 6. Other Analytes Involved with Adipogenesis and Insulin Resistance

Reagents for quantifying the soluble leptin receptor and SDL-1 are not commercially available at this time, although methods for measurement of these analytes in mouse serum have been published (Cohen *et al.* 2002; Cohen *et al.* 2005).

### C. Lipid Metabolism

#### 1. Types and Functions

The four main types of lipids in plasma are free cholesterol, esterified cholesterol, triglycerides, and phospholipids. Lipids are derived from the diet (mainly from long-chain fatty acids), although endogenous recycling (mainly in the liver) occurs. Plasma lipids have poor water solubility; thus, they require water-soluble protein molecules for their transport in plasma. The complex of plasma lipids and proteins are known as apoproteins (also known as apolipoproteins), which contain a core of nonpolar lipids surrounded by a surface layer of phospholipids, free cholesterol, and apoproteins.

Apoproteins are classified according to physical and chemical parameters, and include (in order of increasing density) chylomicrons, very low-density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low-density lipoproteins (LDL), and high-density lipoprotein (HDL). Particle density is proportionally related to the amounts of phospholipid, protein, and triglycerides they contain; increasing particle density corresponds with increasing proportions of phospholipid and protein, and decreasing density corresponds with decreasing proportions of triglyceride. Chylomicrons and VLDL are often referred to as triglyceride-rich lipoproteins. Apoproteins on the surface of the particles serve as ligands for receptors, cofactors of enzyme interaction and structural components (Wagner *et al.* 1999). Table 6-5 lists the mouse apoproteins and describes their known function.

The largest lipoprotein particle is the chylomicron, which transports dietary lipids in the form of triglycerides from the intestines. Chylomicrons contain apoproteins A and B48 and are absorbed into the lymphatics from the intestine, eventually entering the blood. They deliver fatty acids to the tissues with assistance from lipoprotein lipase (LPL) and release glycerol into the blood. As chylomicrons lose triglycerides they become smaller and are called remnants. These remnants acquire apoprotein E from plasma HDL and are rapidly cleared by the liver by the apoprotein E receptor or chylomicron remnant receptor.

VLDL is the second most prevalent lipoprotein particle in normal mouse blood (after HDL) and it transports triglyceride from liver to extrahepatic tissues. VLDL is synthesized in the liver with apoprotein B100, C, and E attached. LPL aids in the release of fatty acids (from triglycerides) to peripheral tissues. LDL, which is present in very low concentrations in normal mice, carries cholesterol to extrahepatic tissues. LDL contains Apoprotein B100 and is the primary source of cholesterol deposited in the intima of arteries in mice. Apoprotein B100 binds to the LDL receptor (LDLR). HDL is synthesized in the liver and contains large amounts of free cholesterol and apoproteins A, C, and E. During metabolism HDL free

TABLE 6-5
MOUSE APOLIPOPROTEINS

| Name | Mol. Wt. | Lipoprotein | Function |
|---|---|---|---|
| ApoA-I | 28,016 | HDL | Structural protein for HDL, activator LCAT |
| ApoA-II | 17,414 | HDL | Structural protein for HDL |
| ApoA-IV | 44,465 | HDL, Chyl | Activator of LPL and LCAT |
| ApoB100 | 545,000 | VLDL, IDL, LDL | Major protein component, binds LDLR |
| ApoB-48 | 261,600 | Chylomicrons, remnants | Structural protein for chylomicrons |
| ApoC-I | 6630 | Chylomicrons, VLDL, HDL | Inhibits binding of LP to the LDLR, LRP, and VLDLR and inhibits CETP activity |
| ApoC-II | 8900 | Chylomicrons, VLDL, HDL | Essential cofactor for LPL |
| ApoC-III | 8800 | Chylomicrons, VLDL, HDL | Inhibits LPL and HL, thus elevates plasma triglycerides |
| ApoC-IV | | | Plays role in triglyceride metabolism |
| ApoE | 34,145 | Chylomicrons, VLDL, HDL, and their remnants | Binds hepatic LDLR and LRP receptors |

HL, hepatic lipase; LCAT, lecithin-cholesterol transferase; CETP, cholesteryl ester transfer protein; LP, lipoprotein lipase; LRP, LDL-receptor related protein.

cholesterol is esterified by the action of lecithin:cholesterol acyltransferase (LCAT). The exchange of cholesterol ester for triglycerides (from VLDL) results in a less dense HDL subfraction that is completely removed from the circulation by the liver. Exchange of esterified cholesterol for triglycerides is mediated by cholesteryl ester transfer protein (CETP) in humans; however, this activity is absent in mice. Phospholipid transfer from VLDL to HDL is mediated by phospholipids transfer protein (PLTP). High levels of soluble hepatic lipase are found in mouse plasma (Lusis, 2000; Wagner *et al.* 1999). HDL is thought to transport cholesterol from peripheral tissue to liver by a process known as reverse cholesterol transport (RCT). This process is believed to be facilitated by the adenosine triphosphate (ATP)-binding cassette transporter A1 (ABCA1), which transports phospholipids and cholesterol to the acceptors apoprotein A-1 and apoprotein E (Aiello *et al.* 2002). HDL also serves as a reserve of apoprotein C and apoprotein E necessary for VLDL metabolism. Table 6-6 lists the apolipoprotein receptors found in mice and describes their ligands and functions.

### 2. Atherosclerosis

Atherosclerosis is a major factor in heart disease, stroke, and peripheral vascular disease in humans, and as such, is the principal cause of death in western countries. The etiology is complex, involving both genetic and environmental components. Although there are some examples of single gene defects in humans that lead to atherosclerosis, these conditions are rare and do not explain disease prevalence.

Atherosclerosis is characterized by the formation of plaques in the intima of large and medium size arteries, usually in locations where blood flow is disturbed. The plaques contain a variety of cells including endothelial cells, monocytes or macrophages, smooth muscle cells, and lymphocytes, which secrete products that modulate progression of plaque formation. In addition plaques contain a complex mix of collagen, proteoglycans, occasional cartilaginous tissue with calcification and lipoprotein (primarily LDL). Disease risk correlates directly with elevated circulating levels of LDL cholesterol and risk is indirectly correlated to levels of HDL. Thus, although LDL promotes atherosclerosis, HDL protects against it (National Cholesterol Education Program 1993). The combination of high LDL and low HDL is termed dyslipidemia and is found in human patients with insulin resistance syndrome.

Mice do not develop spontaneous atherosclerosis, because they have low circulating levels of LDL and lack CETP activity. However, genetically engineered mice that over- or under-express genes involved in lipid metabolism have contributed greatly to our understanding of lipoprotein metabolism (Marschang and Herz 2003). In particular, transgenic mice have been developed with plasma lipid profiles that are similar to those of humans with atherosclerosis (Breslow 1994, 1996).

In humans and transgenic mice with elevated LDL (or low HDL), LDL passively diffuses across the endothelium, especially in areas where endothelial cell morphology has been altered by the sheer forces generated by blood turbulence. Once LDL is within the intima there is an interaction between LDL-apoprotein B and matrix proteoglycans resulting in LDL trapping. LDL undergoes modifications associated with oxidation, lipolysis, proteolysis, and aggregation that contribute to inflammation and uptake of LDL by tissue macrophages. Lipoxygenases (LOs) insert molecular oxygen into polyenoic fatty acids producing hydroperoxyeicosatetraenoic acid (HETE). Endothelial cells release HETE into the vessel wall, where it initiates oxidation of LDL. Further oxidation of LDL is aided by myeloperoxidase and sphingomyelinase. Mice lacking LOs have diminished atherosclerosis. Macrophages and monocytes recognize oxidized LDL via their surface scavenger receptors and become loaded with cholesterol ester, forming a fatty streak appearing along the vessel wall. HDL, on the other hand, removes excess cholesterol from peripheral tissue and inhibits lipoprotein oxidation. HDL carries paraoxonase, which

TABLE 6-6
MOUSE APOLIPOPROTEIN RECEPTORS

| Name | Expressed | Ligands | Function | Regulators |
|---|---|---|---|---|
| Low density lipoprotein receptor (LDLR) | Widely | apoB100, apoE | Internalizes and degrades particles | SREBPs |
| LDL receptor–related protein-1 (LRP1) | Widely (highest in liver and brain) | apoE, diverse spectrum | Likely candidate for chylomicron remnant receptor | |
| Very low-density lipoprotein receptor (VLDLR) | Heart, skeletal muscle, adipose, not liver | Triglyceride rich lipoproteins (chylomicrons, VLDL) | Important in adipose tissue metabolism; VLDLR$^{-/-}$ do not develop obesity; role in neuronal migration in cortex and cerebellum | |
| **Scavenger receptors** | | | | |
| SR-AI | Homotrimeric macrophages | Modified (oxidized) LDL | Involved in atherosclerotic lesions | |
| SR-AII | Homotrimeric macrophages | | | |
| CD3b | | Bind native and modified LDL and HDL | Involved in atherosclerotic lesions; a long-chain fatty acid transporter and receptor for the matrix glycoprotein, thrombospondin-1 | |
| SR-BI | Liver steroidogenic tissues | Selective HDL-cholesterol uptake | Upregulated by oxidized LDL by PPAR-γ; role in reverse cholesterol transport | |

SREBP, sterol regulatory element-binding protein.

degrades oxidized phospholipids (Lusis 2000) and protects against subsequent oxidative damage.

Oxidized LDL stimulates endothelial cells to express adhesion molecules (such as ICAM, VLA-4, and VCAM) and monocyte colony-stimulating factor (M-CSF) on their luminal surface, which attracts additional monocytes and lymphocytes. Oxidized LDL also inhibits nitric oxide production. Infiltrating thymic-derived lymphocytes (T cells) and macrophages initiate migration of smooth muscle cells from the media to the intima, where they synthesize matrix components. MCP-1 is also released by macrophages, inducing monocyte differentiation, migration, and scavenger receptor expression.

The accumulation of excess free cholesterol can be inhibited by activation of acyl CoA:cholesterol acyltransferase (ACAT)-mediated cholesterol esterification and cellular cholesterol efflux. One mechanism responsible for cholesterol efflux is secretion of apoprotein E by macrophages that promotes efflux via HDL. If this fails, as in the case of cholesterol-laden macrophages in plaques, apoptosis of the macrophages ensues. Loading of the endoplasmic reticulum with free cholesterol activates ER resident protein kinase and the unfolded protein response (UPR) that initiates apoptosis through activation of caspase-12 (Feng *et al.* 2003a).

As macrophages undergo apoptosis they create a necrotic core in the plaque, promoting extracellular cholesterol cleft formation. Smooth muscle cells create a fibrous cap over the plaque near the luminal surface. The lesion advances as T cells, activated by CD40-CD40L ligation, release cytokines such as interferon-γ (IFN- γ) and TNF-α. These substances activate matrix degrading proteases and adhesion molecules, promoting additional inflammation. As the inflammation progresses the fibrous cap becomes compromised, leading to physical rupture and the generation of a thrombogenic surface. Oxidized LDL increases the production of tissue factor that, on the thrombogenic surface, initiates the coagulation cascade and thrombus formation. Each of these details have been elucidated using genetically modified mice (Auerbach *et al.* 1992; Choudhury *et al.* 2004; Feng *et al.* 2003b; Lusis 2000; Reardon and Getz 2001; Tailleux *et al.* 2003; Trigatti *et al.* 2004). For a further discussion of these models readers are urged to read Chapter 16 in this book.

### 3. Measurement

For measurements of plasma lipids, serum is the preferred sample. Serum can be stored at 4°C for 5–7 days without adverse effect on measurements.

a. SERUM CHOLESTEROL AND TRIGLYCERIDES Serum cholesterol in mice can be measured using the enzymatic oxidation method of Roschlay (Meade and Gore 1982), the Lipid Research Clinic Program protocol (Morrisett *et al.* 1982), or the Abell technique (Mitruka and Rawnsley 1977). The most common enzymatic method employs cholesterol ester hydrolase (to convert cholesterol ester to cholesterol), cholesterol oxidase (which oxidizes cholesterol to hydrogen peroxide and

cholest-4-en-3-one) and peroxidase (which catalyzes a reaction involving hydrogen peroxide, phenol and 4-aminoantipyrine to form the dye quinonelmine). All of these components of this test are combined in a single reagent mix. The dye absorbance is measured at 500 nm (Choudhury *et al.* 2004).

Triglyceride (TG) levels, which are mainly reflective of the TG content of chylomicrons and VLDL in the mouse, are quantified using a variety of enzymatic methods. The most popular method combines lipoprotein lipase, glycerol kinase, and glycerol-phosphate oxidase with peroxidase and 4-aminoantipyrine (usually in two reagents). In this method, serum TGs are converted to glycerol by LPL via hydrolysis. Next the glycerol is phosphorylated in an ATP-dependent reaction catalyzed by glycerol kinase. Glycerol-phosphate is catalyzed to dihydroxyacetone and hydrogen peroxide by glycerophosphate oxidase and peroxidase catalyzes the final reaction in which peroxide and 4-aminoantipyrine are converted to a stable dye and absorbance can be read at 500 nm (Tsimikas *et al.* 2000). Reagents for both total cholesterol and total TGs may be used in an automated analyzer or purchased directly from a chemical company with instructions for manual laboratory analysis.

Total serum cholesterol and TG levels vary by strain, gender, age, length of fast, and diet. Jiao *et al.* (1990) studied total plasma cholesterol (TPC) and TG levels of various inbred strains fed a standard diet. After 18–20 h of fasting, TPC levels ranged from 55 mg/dl (AKR/J) to 128 mg/dl (NZB/B1NJ), and TG levels ranged from 13 mg/dl (C57BL/6) to 67 mg/dl C3H/HeJ; each much lower than seen in normal humans. Albers and Piagen (1999) quantified total cholesterol levels in 15 strains give a standard laboratory diet and fasted for 4 h before blood collection. Levels (in 6- to 8-week-old mice) varied greatly between strains with C57BLKS/J females having the lowest level (39 mg/dl) and NZB/B1NJ males having the highest levels (127 mg/dl). For a given strain, males always had higher levels than females. Trends in total HDL levels in the same strains paralleled total cholesterol levels. Triglyceride levels also varied greatly among strains but did not parallel cholesterol levels. Among the strains tested, C57BL/6J mice had the lowest TG levels (71–80 mg/dl) and C3H/HeSnJ the highest (F, 162 mg/dl; M, 231 mg/dl). Differences between genders of the same strain were less pronounced, compared to cholesterol and males generally had higher levels than females.

A transient cause of plasma lipid elevation (hyperlipidemia) in the mouse is related to recent feeding (postprandial hyperlipidemia), especially on a high-fat diet. Causes of persistent hyperlipidemia include diabetes, sustained feeding of a high-fat diet, and nephrotic syndrome.

b. HDL, LDL, IDL, AND VLDL Levels of individual apolipoproteins can be measured by density gradient ultracentrifugation combined with either electrophoretic, immunologic, chemical, or morphologic analyses. Although published reference ranges are not available, the distribution and characterization of murine apoproteins has been reported (Camus *et al.* 1983). Similar methods were used to evaluate plasma apoproteins in mice consuming atherogenic diets (Morrisett *et al.* 1982). More recently the profile of murine plasma lipoprotein cholesterol has been determined by fast protein liquid chromatography with on-line post-column analysis of Superose 6 gel-filtration eluates (Sehayek *et al.* 2003; Strauss *et al.* 2001).

In contrast to humans, mice normally have low levels of LDL in their plasma (15 mg/dl), with the major plasma lipoprotein being HDL. The low LDL concentration is due to editing of 70% of apoprotein B mRNA transcripts in mouse liver leading to apoprotein B48 containing particles that are cleared much faster than LDL in humans (Lusis 2000). In contrast, human apoprotein B mRNA transcript editing only occurs in the intestine.

A much simpler methodology has been developed to measure HDL and non-HDL lipoproteins in mice, based on precipitation and enzymatic analysis. When phosphotungstate and magnesium salt is added to serum (or plasma), all lipoprotein, except HDL, is precipitated and can be removed. The remaining solution is then tested for cholesterol as described previously. The non-HDL-cholesterol component is calculated by subtracting HDL-cholesterol from total cholesterol in the nonprecipitated sample (Sehayek *et al.* 2003). Some strains develop elevated cholesterol levels associated with increases in non-HDL levels after consuming high fat diets (Breslow 1994).

c. MOUSE APOPROTEINS Commercial antibodies against mouse apoprotein A1 and apoprotein A2 are available and ELISAs have been described for the measurement of these apoproteins in plasma and in HDL fractions of plasma (Dansky *et al.* 1999). Plasma apoprotein A1 levels can also be measured by multiplex analysis.

An assay for mouse apoprotein J (ApoJ or clusterin) has also been described (Navab *et al.* 1997). ApoJ, which is a ubiquitous glycoprotein postulated to have multiple functions, is associated with HDL and is the amyloid-associated protein associated with amyloid plaque formation in Alzheimer's disease in humans.

d. OTHER ANALYTES ASSOCIATED WITH LIPID METABOLISM AND ATHEROSCLEROSIS IN MICE ELISA kits are commercially available for the quantitation of many mouse coagulation proteins including: fibrinogen, factor VII, d-dimer, tissue factor, and von Willebrand's factor antigen. ELISA kits are also available to quantify many murine products of arachidonic acid metabolism including HETE. Reagents, ELISA kits, and multiplex assays are available for murine inflammatory cytokines and chemokines (see the "Cytokines and Chemokines" section), as well as monocyte colony stimulating factor. Dansky *et al.* (1999) have described assays for the quantitation of murine aryl esterase and paraoxonase. Table 6-7 lists the mouse enzymes involved in lipid metabolism with references that describe their measurement.

## D. Immunoglobulins and Immune Function

The innate and adaptive immune responses have been extensively studied (see Volume 4 of this series) and therefore we

TABLE 6-7
MOUSE ENZYMES INVOLVED IN LIPID METABOLISM

| | |
|---|---|
| Lipoprotein lipase (LPL) Strauss *et al.* 2001 | Hydrolysis of triglycerides carried by chylomicrons and VLDL and subsequent uptake of FFAs in extrahepatic tissues |
| Hepatic lipase (HL) Homanics *et al.* 1995 | Involved with HDL metabolism where it hydrolyses triglycerides and phospholipids; enhances uptake of cholesterol esters by hepatocytes |
| Hormone-sensitive lipase (HSL) Osuga *et al.* 2000 | Hydrolyzes neutral lipids in peripheral organs supplies mice with FFAs in the postabsorptive state |
| Lecithin-cholesterol transferase (LCAT) Mehlum *et al.* 1995 | Accounts for most of the cholesterol ester synthesis in plasma; aids in maturation of lipoproteins, especially HDL |
| Cholesteryl ester transfer protein (CETP) | Mediates the exchange of cholesterol esters from HDL to triglyceride-sick lipoproteins (VLDL) in exchange for triglycerides; mice have no activity in plasma |
| Phospholipid transfer protein (PLTP) Jiang *et al.* 1999 | Mediates transfer of phospholipids from triglyceride-rich lipoproteins to HDL; remodels HDL |
| Endothelial lipase (EL) Jaye and Krawiec 2004 | Promotes turnover of HDL components and increases the catabolism of apolipoprotein A1 |

will not attempt to describe all the features of mouse immunity in detail here. Readers are directed to Volume 4, Molecular and Cellular Immunology of the Mouse, for a general overview, and the 15 chapters that follow for a more detailed description. This section describes the growing number of quantifiable soluble serum proteins and lipids associated with immunity and inflammation in the mouse (see Table 6-3). Furthermore, in addition to immunodeficiency, autoimmunity, and allergy, investigations of atherosclerosis, obesity, diabetes, cancer, as well as infectious diseases, each have immunologic and inflammatory components.

#### 1. Immunoglobulins (Ig)

As in humans, mouse immunoglobulins (Ig) are molecules composed of four polypeptide chains; two of lower molecular weight called light (L) chains, and two with higher molecular weight called heavy (H) chains. Disulfide bonds link one L chain to one H chain, and the two H chains to each other. Immunoglobulin H chains are composed of four to five domains, including an N-terminal variable region domain and four constant-region domains. In addition, structural differences in the constant-region domains of the heavy chain are used to classify the five different classes of immunoglobulin, IgM, IgG, IgA, IgE, and IgD. L chains are composed of only two domains and structural differences in these domains are used to classify L chains as either kappa or lambda type. In the mouse, 95% of serum Ig has kappa L chains. Any individual antibody secreting B cell (or plasma cell) will make a single Ig molecule composed of two identical H chains and two identical L chains. Mice make four subtypes of IgG: IgG1, IgG2a, IgG2b, and IgG3. Certain strains, C57BL/6, C57Bl/10, SJL, and NOD, do not make IgG2a but rather make a novel IgG2c. The IgG subtypes in mice are not exact homologues of human subtypes (Mestas and Hughes 2004).

Prenatal (transplacental) transfer of maternal Ig as well as postnatal transfer across the intestinal epithelium is limited to the IgG2a, 2b, and 3 subclasses of immunoglobulin and is mediated by neonatal Fc receptors (FcRn) located in the placenta or on the intestinal brush border of the proximal small intestine (Bankert and Mazzaferro 1999). Mouse IgG2b fixes complement by the classical pathway and IgG1 and IgG2a fix complement by the alternative pathway. IgE and IgG1 are homocytotropic antibodies capable of binding to receptors on mast cells and basophils and mediate immediate hypersensitivity reactions. Although IgG and IgE circulate in the mouse as monomers, IgM circulates as a pentamer and IgA circulates as a polymeric molecule. Normally very little IgD can be detected in serum.

Serum levels of IgM, IgA, IgG, and IgE are influenced by the rate of synthesis and rate of catabolism. Like humans, the rate of IgG catabolism in mice is directly proportional to the serum concentration of the subclass. The average half-life of murine IgG is 4.5 days. The catabolic rate of IgA is independent of serum concentration. Mice synthesize from 50–130 mg/kg/day of total Ig, although this is dependent on strain and level of antigenic stimulation.

Certain strains have a propensity to develop specific T-helper (TH, CD4+) cell subclasses in response to antigenic stimulation, and these strains are referred to as having principally a TH1 or TH2-like phenotype. Typically CD4+ lymphocytes modulate immune responses by the cytokines they secrete. Those secreting IL-1, IFN- $\gamma$, and lymphotoxin are generally referred to as TH1 (and are favored responses for immunity against viruses and intracellular pathogens) and those secreting IL-4, IL-5, IL-10, and IL-13 are referred to as TH2 (and enhance humoral immunity while suppressing cell mediated immunity). When confronted with the same antigen, BALB/c mice exhibit a TH2-dominant response, and C57BL/6 mice exhibit a TH1-dominant response. IL-4 participates in immunoglobulin (antibody) class switching (see Volume 4, chapter 5). Consequently, TH2 strains are the models of choice for investigations of allergic inflammation because they produce higher concentrations of IL-4 induced immunoglobulin classes (IgE and IgG1).

The CBA/N strain is deficient in its ability to produce IgM and IgG3. Immunoglobulin levels are also greatly reduced in germ-free mice, offspring of mice on zinc-deficient diets, and mice on protein-deficient diets (Quimby 1999b).

Quantifying the various classes and subclasses of murine Ig can be done using various immunoassays including: radial immunodiffusion, RIA, or enzyme immunoassay. Both RIA and enzyme immunoassay have the higher degrees of sensitivity

that are needed to accurately quantitate levels of IgE and IgD in murine serum. Enzyme immunoassay has become the favored assay to avoid isotope handling and disposal. The reported concentrations of normal BALB/c mice are: IgG1 (6.5 mg/ml), IgG2a (4.2 mg/ml), IgG2b (1.2 mg/ml), IgG3 (0.1–0.2 mg/ml), IgA (0.7 mg/ml), IgM (1.0 mg/ml), and IgE and IgD are both less than 0.01 mg/ml (Bankert and Mazzaferro 1999). In addition, there are many commercial sources of enzyme-linked antibodies that supply reagents (and instructions) for developing assays to measure antigen-specific antibody concentrations by subclass of antibody.

### 2. Complement

The complement system is composed of 40 or more chemically and immunologically distinct proteins capable of interacting with antibodies, certain bacterial products, and cell membranes. A brief summary of this system is described later. Please refer to two recent publications (Quimby 1999a; Turnberg and Botto 2003) for more details about the structural and functional aspects of each protein of the complement system. The role of the complement system in mouse immunity is described in Volume 4 (Overview) of this series.

The sequential activation of individual complement proteins from inactive to active substances is a dynamic event called the complement cascade. The ability of the first component of complement, C1, to bind specific sites on the heavy chain of mouse IgG2b and activate a sequence of reactions leading to production of a molecular unit capable of lysing a target cell membrane has established the complement system as the primary mediator of antibody-antigen reactions. Recent findings suggest that the pentraxins, C-reactive protein (CRP), serum amyloid protein (SAP), and pentraxin 3 (PTX3) can bind to complement component Clq and activate the classical pathway. This may be an important mechanism for removal of apoptotic cells that might otherwise predispose to autoimmune disease (Nauta *et al.* 2003). Each protein of the complement system is normally present in the circulation as an inactive molecule.

Although the complement cascade may be activated by any of four separate pathways, the central event for each is activation of C3 to C3b yielding a small C3 $\alpha$ chain fragment. The two C3 convertases are C4b2a (for the classical, lectin, or pentraxin pathways) and C3bBb for the alternative pathway. Each C3 convertase cleaves C3 and adds the C3b fragment to the convertase complex forming C5 convertase. Cleavage of C5 leads to the membrane attack complex (MAC) common to all activation pathways (Goldsby *et al.* 2003). Assemblage of the MAC on the surface of a target cell leads to the formation of a large channel enabling ions and other small molecules to diffuse out leading to cell death. The activated components of complement also participate in chemotaxis, phagocytosis, cell adhesion, and B-cell differentiation.

There are many notable differences between mice and humans regarding expression of complement components and regulation of cascade activation. Mice have both an active and inactive form of circulating C4 and the genes (both on chromosome 17) are designated *Ss* and *Slp*, respectively. Many inbred strains do not synthesize active C5 (e.g., DBA/2 and A/J strain) due to a post-translational defect and therefore they cannot generate a MAC. Some strains are deficient in the production of C8 (e.g., DBA/2J strain). Complement component 6 exists as two allelic forms in mice with 90 and 100 kDa molecular weights.

Similarly there are several notable differences in the regulators of complement activation. Most of these regulators are found in the regulator of complement activation (RCA) locus on murine chromosome 1 and, as in humans, restrict assembly and stability of convertase enzymes. In mice, decay acceleration factor (DAF) is encoded by two genes. However, only the product of *Daf-1* is widely dispersed on all tissues. This membrane-anchored protein inhibits C3 cleavage by accelerating decay of C3 convertases. In mice it is the major regulator of C3 in the skin (not kidney) and is a ligand for CD97 which, on cross-linking, leads to lymphocyte activation. CD59 is a membrane-anchored inhibitor of C5b-9 formation (MAC) and prevents C9 from binding the C5b-8 complex. Deficiencies of CD59 in humans lead to hemolytic anemia but not in mice. Membrane cofactor protein (MCP) is a major cofactor for factor I in humans causing cleavage of C3b and C4b deposited on self-tissue. In mouse MCP is only expressed in the testis. Complement receptors 1 and 2 are encoded by separate genes in humans but are produced by alternative splicing of a single gene in mouse. Mice have a complement receptor 1 (CR-1) related gene/protein Y (Crry) not found in humans, which is a membrane-anchored C3 inhibitor. It is the major regulator of C3 in mouse kidney and has some of the functions as MCP and CR1 in humans. Crry has DAF activity and serves as a cofactor for factor I (which enzymatically cleaves C3b).

The mouse has been widely used in studies on the biosynthesis and molecular biology of individual components of complement. The genes encoding 39 components, subcomponents, receptors, and inhibitors have been identified in mice.

The complement components may be quantified by assays designed to measure the functional properties of these proteins or their antigenic properties. Tests designed to measure antigenic properties of complement are generally simpler, less subject to error, and less expensive; however, they have the disadvantage of measuring both active and inactive forms of their proteins and therefore may not correlate well with functionally active protein. Functional assays measure the ability of the entire classical or alternative pathway, or individual components of pathways to lyse (hemolyze) antibody-coated (sensitized) or noncoated (for the alternative pathway) red cells in suspension or in agarose gel. These assays are precise and sensitive (Quimby 1999a).

Antigenic assays include radial immunodiffusion, electroimmunodiffusion (rocket electrophoresis), automated immunoprecipitation, crossed immunoelectrophoresis, and more recently ELISA (Quimby 1999a). ELISA kits are

commercially available for quantifying murine C1q, C3, C4, C3a (desArg), and C3a. In addition antibodies are commercially available for these as well as murine C4a, C4d, C5, and C6. Complement components C2, C5, C6, C7, and Factor B may be quantified using functional assays (Quimby 1999a). Membrane-bound complement regulators, Crry, CD59, and DAF, can all be detected using previously published antibodies (Lin *et al.* 2001, 2002).

The principle mediator of the lectin activation pathway is mannose-binding lectin (MBL). After binding to mannose residues on the surface of microorganisms, two MBL-associated serine proteases (MASPs), MASP-1 and MASP-2, bind to MBL. This complex causes cleavage and activation of C4 and C2 (MASP1 and 2 mimic the activities of Cl4 and Cls). Commercially available ELISA kits are available for murine MBL-A and MBL-C quantitation.

Other activators of complement include members of the pentraxin family. In mice this may include CRP or SAP, although the concentration of CRP in normal mice is very low. Immunologic reagents are available to quantify both pentraxins in mice (Gentry 1999; Quimby 1999b).

### 3. Circulating Immune Complexes (CIC)

Circulating immune complexes (CIC) are multimolecular substances composed of antigen, antibody, and activated complement components. In mice, IgM, IgG1, IgG2a, and IgG2b have complement activation regions. Although CIC may form following exposure to circulating foreign antigens, such as those associated with microorganisms, they are also common manifestations of spontaneous autoimmune disease such as that seen in (NZB X NZW)F1, MRL/Lpr, BXSB, and KRN strains. CIC are cleared from the circulation by both CR-1, CR-2, and FcR. Tissue deposition of immune complexes may lead to vasculitis. CIC have been quantified in mice by capitalizing on their binding to various complement receptors, by precipitation of Clq with polyethylene glycol, or by immunoassay. Commercially available ELISA kits are commonly employed today (Quimby 1999b).

### 4. Autoantibodies

More than 100 inbred strains or mutant lines spontaneously develop autoimmune disease or are susceptible to autoimmune disease induction. The details of many of these lines may be found in Volume 4, Chapters 11 and 12 in this series. Autoimmune diseases in mice include: thyroiditis, rheumatoid arthritis, Sjögren's syndrome (SS), hemolytic anemia, lupus erythematosus, type 1 diabetes mellitus, experimental allergic encephalitis, oophoritis, orchitis, gastritis, ulcerative colitis, and polyendocrine disease (Boyton and Altmann 2002; Ravirajan and Isenberg 2002; Sakaguchi 2000).

Antibodies directed to self-antigens in the mouse have been quantified using a wide range of methods from immunofluorescence to ELISA. Table 6-3 lists the commercially available ELISA kits used to quantify murine autoantibodies. Those antigenic targets associated with systemic lupus erythematosus include: cardiolipin, double-stranded deoxyribonucleic acid (dsDNA), single-stranded deoxyribonucleic acid (ssDNA), histone, β-2 glycoprotein, proliferating cell nuclear antigen (PCNA), neutrophil cytoplasmic antibody (cANCA), ribosomal P, and Smith. Antigenic targets for arthritis include: rheumatoid factor (RF), collagen type 1, collagen type 2, and ssDNA. Antibodies against insulin and glutamic acid decarboxylase are seen in type 1 (juvenile) diabetes. Mixed connective tissue diseases are characterized by antibodies to ribonuclearprotein (RNP). Antibodies to myeloperoxidase (MPO) may be observed in vasculitis. Mice with SS develop autoantibodies to SS antigens A and B (SSA and SSB, respectively). Panels containing 14 autoantigens are available as multiplex assays for quantifying murine autoantibodies.

### 5. Cytokines and Chemokines

a. INTERLEUKINS (IL) (ILs are cytokines that are secreted by leukocytes and act on other leukocytes. Interleukins have been classified based on the secretory cell type (i.e., monokines vs. lymphokines), and they have been classified based on whether they are primarily involved in innate (IL-1, IL-6, IL-12, TNF-α, IFN-α), or adaptive (IL-2, IL-4, IL-5, IL-10) immunity. Commercially available ELISA kits are available to quantify most murine interleukins, as demonstrated in Table 6-3.

***i. INTERLEUKIN-1 (IL-1)*** IL-1 is a name for two proteins, IL-1α and IL-1β, that are encoded by separate genes. Along with IL-1 receptor antagonist, IL-18, IL-6, and TNF-α, these proteins modulate acute inflammation. The effects of IL-1 are pleotrophic and involve bone remodeling, insulin secretion, appetite regulation, fever induction, neuronal development, and many others. Both IL-1α and IL-1β are secreted as 269–271 amino acid (aa) pro-cytokines that are enzymatically cleaved into bioactive 17-kDa segments. Unlike IL-1β, the intact procytokine of IL-1α is also bioactive, both within the cytoplasm and on the cell surface, where it is anchored to the cell membrane via a mannose glycosylation residue that attaches to the membrane-associated lectin. There is 78% sequence identify between mouse and human IL-1β genes and 58% identity between mouse and human IL-1α genes. A third gene encodes IL-1 receptor antagonist, a soluble 25-kDa molecule with 19% sequence homology to IL-1α and 26% homology with IL-1β. Mouse IL-1ra is 75% homologous with human IL-1ra.

There are two IL-1 receptors, types I and II, but only IL-1RI is capable of signal transduction. IL-1ra inhibits the action of IL-1α and IL-1B by binding IL-1RI. A 60-kDa form of IL-1RI has also been described that is soluble and preferentially binds IL-1ra. IL-1RII has no signal transducing associated protein and serves to modulate levels of IL-1α, IL-1β, and IL-1ra by binding them on the cell surface. It can also occur as a soluble receptor. With the recent finding of six new members in the IL-1 ligand family (in humans), a revised nomenclature for both IL-1 ligands

and IL-1/IL-18 receptor families has been developed (Sims 2002). Further details may be found in Volume 4 (Overview and Chapter 8) of this series.

*ii.* **Interleukin-2 (IL-2)** *IL-2* is a lymphokine secreted by activated T-helper cells. It acts in an autocrine fashion to induce the expression of IL-2 receptor on T cells, resulting in T-cell proliferation (Cogoli-Greuter *et al.* 2004). IL-2 also acts in a paracrine fashion modulating the activities of B cells, natural killer (NK) cells, and lymphocyte activated killer (LAK) cells. IL-2 is a glycoprotein of 133 amino acids (in humans) with 63% homology between mouse and human. The IL-2 receptor (IL-2R) is a multisubunit cellular receptor belonging to the class 1 cytokine receptor family (hematopoietin receptor family). The IL-2R has α-, β-, and γ-chains. β- and γ-chains interact to transduce the IL-2R signal and the γ-chain is shared with receptors for IL-4, IL-7, IL-9, and IL-15.

*iii.* **Interleukin-3 (IL-3)** IL-3 is also known as mast cell growth factor. It is produced primarily by activated T cells and stimulates differentiation of pluripotent hematopoietic stem cells, mast cells, and dendritic cells. Murine IL-3 is a comitogenic stimulus for stem cell factor dependent mast cells, inducing formation of mast cell–specific secretory granules and mast cell growth (Austen and Boyce 2001). Mouse IL-3 is only 29% homologous with the human protein, and there is no cross-species activity.

*iv.* **Interleukin-4 (IL-4)** IL-4 is a pleiotropic cytokine produced by activated T cells, mast cells, and basophils. It has multiple effects on B cells, T cells, monocytes, mast cells, myeloid and erythroid progenitors, fibroblasts, and endothelial cells. IL-4 induces immunoglobulin class switch to IgE and IgG1 in mice and is responsible for the downstream events leading to differentiation and activation of TH2 cells. IL-4 also induces expression of adhesion molecules like VCAM, TH2 cytokines such as IL-5, IL-6, and IL-9 and chemokines like eotaxin-1 and -2. IL-4 primes mast cells and basophils leading to enhanced activation during allergic challenge (Mueller *et al.* 2002). Homology between mouse and human molecules is low (25%) and each is species-specific in its biologic activity.

*v.* **Interleukin-5 (IL-5)** IL-5 is produced by T cells and induces the differentiation of activated B-2 cells into Ig secreting cells. It induces the growth of B-1 progenitors and IgM production by B-1 cells. IL-5 induces class switch, favoring production of IgA, IgG1, and IgE. On eosinophils, IL-5 induces IgA and IgG receptors and stimulates leukotriene (LT), C4, and PAF secretion, in addition to inducing eosinophil growth and maturation. The receptors for IL-5 consist of a ligand binding α-subunit and a non–ligand binding (common) signal transducing β-subunit that is shared by receptors for IL-3 and granulocyte-monocyte colony-stimulating factor (GM-CSF) (Sato and Miyajima 1994).

*vi.* **Interleukin-6 (IL-6)** IL-6 is secreted by a wide variety of cells including T cells, B cells, monocytes, fibroblasts, hepatocytes, keratinocytes, astrocytes, and endothelial cells. It has broad pleiotropic effects on host defense, acute phase responses, immune responses, and hematopoiesis. IL-6 is classified as an inflammatory cytokine and based on a helical cytokine structure and subunit makeup, IL-6 is the prototypic member of a family of molecules that includes leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), cardiotrophin (CT-1), and IL-11. Mouse IL-6 is 25 kDa and contains four cysteines and contains *O*-glycosylation sites and shares 40% homology with the human molecule (Van Snick 1990). The IL-6 receptor has two subunits, a nonsignal transducing subunit binding with low affinity (α-subunit), and a signal transducing subunit (β-subunit) that does not bind IL-6 by itself but participates in high-affinity binding. The soluble IL-6Rα chain binds IL-6 and the complex induces expression of MCP-1, which attracts monocytes into areas of inflammation (Kaplanski *et al.* 2003).

*vii.* **Interleukin-7 (IL-7)** IL-7, previously called lymphopoietin-1, is expressed by stromal cells, especially in the bone marrow and thymus, where it promotes thymopoiesis of T cells and the differentiation of pro-B cells into pre-B cells (Aspinall *et al.* 2004; Goldsby *et al.* 2003). Mouse IL-7 has 65% amino acid sequence homology with human IL-7 and both proteins exhibit cross-species activity.

*viii.* **Interleukin-8 (IL-8)** IL-8 is not expressed in the mouse; however, another protein, KC, is secreted and has many properties of the human chemokine, GRO, which is known to bind the IL-8 receptor (see the "Cytokines and Chemokines" section).

*ix.* **Interleukin-10 (IL-10)** IL-10 is the prototypic member of the IL-10 cytokine family comprising IL10, IL-19, IL-20, IL-22, IL-24 (FISP), and IL-26. IL-10 is a 178 amino acid protein with an 18 amino acid signaling sequence. Both mouse and human IL-10s have two intrachain disulfide bonds and form non–disulfide linked homodimers. Mouse and human IL-10 are 72% homologous. IL-10 is a TH2 cytokine, which inhibits IFN-γ and GM-CSF production by TH1 cells. Additionally it induces $CD8^+$ T-cell chemotaxis, inhibits T-cell apoptosis, participates in IgA class switch in B cells, induces histamine release from mast cells, and promotes TNF-α and GM-CSF production by NK cells. IL-10 inhibits secretion of the neutrophil chemokines MIP-1α and MIP-1β and blocks production of IL-1β and TNF-α by neutrophils. It is immunosuppressive to dendritic cells and induces the differentiation of a subset of regulatory $CD4^+$ T cells (Tr1) (Grouz and Cottrez 2003; Morel *et al.* 1997).

*x.* **Interleukin-11 (IL-11)** IL-11, also known as adipogenesis inhibitory factor (AGIF), is a pleiotropic cytokine with effects that overlap that of IL-6. IL-11 is a member of the IL-6 cytokine family and as such has a four-helix bundle fold motif. It binds to the multimeric IL-11 receptor that shares the promiscuous gp130 signaling β-subunit with other receptors in this family. The IL-11R α chain is unique and binds IL-11 but does not have a cytoplasmic domain; instead binding leads to homodimerization of the β-chain that activates the Janus kinases. IL-11 stimulates proliferation and differentiation of

monocytes and megakaryocytes causing thrombopoiesis. It also activates osteoclasts and enhances bone resorption, decreases new bone formation, and stimulates chondrocyte and synoviocyte production. IL-11 protects small intestinal epithelial cells from chemotherapy and radiation injury and ameliorates inflammatory bowel disease (Schwertschlag *et al.* 1999). IL-11 inhibits adipogenesis, regulates neuronal differentiation, and regulates T-cell function (enhances TH2 and inhibits TH1 cytokine production). Overexpression of IL-11 in the lung causes airway remodeling, fibrosis, and mononuclear nodules analogous to the clinical picture in chronic asthma. IL-11 is also required for the uterine decidualization response (Robb *et al.* 2002; Zheng *et al.* 2001).

***xi. Interleukin-12 (IL-12)*** IL-12, also known as natural killer cell stimulatory factor (NKSF), is a heterodimeric cytokine composed of a 40-kDa (p40) subunit and a 35 kDa (p35) subunit. The p40 subunit is shared by IL-23, a cytokine with similar activities. Macrophage, monocytes, and dendritic cells produce IL-12 after activation of Toll-like receptors (TLR) on these cells by bacterial ligands. IL-12 induces production of IFN- $\gamma$ by TH1 and NK cells and intact IL-12 skews the balance between TH subsets in favor of TH1 cells. IL-12 binds to the IL-12 receptor that is composed of two subunits, $\beta$1 and $\beta$2, on the surface of NK and TH1 cells. IL-12p40 interacts with IL-12R$\beta$1, and IL-12p35 binds IL-12R$\beta$2. Negative feedback regulation of IL-12 production involves down regulation of TLR signaling by phosphoinositide 3-kinases (PIKs). Thus IL-12 is centrally involved at the interface of innate and adaptive immunity (Fukao and Koyasu 2003: Ottenhoff *et al.* 2002).

***xii. Interleukin-13 (IL-13)*** IL-13, along with IL-4 and IL-5, is a member of the type-2 cytokine family and as such is involved in inflammation, mucus production, tissue remodeling, and fibrosis. This single-chain protein shares 58% amino acid sequence homology with human IL-13. IL-13 is produced by activated T cells, mast cells, and NK cells and promotes TH2 responses including synthesis of IgE. Signaling is mediated by the type-2 IL-4 receptor which consists of IL-4R$\alpha$ and IL-13R$\alpha$1 chains. Another IL-13 binding protein, IL-13R$\alpha$2, strongly inhibits the activity of IL-13 (Goldsby *et al.* 2003; Mentink-Kane and Wynn, 2004).

***xiii. Interleukin-17 (IL-17)*** IL-17, also known as cytotoxic T lymphocyte–associated antigen-8 (CTLA-8), is produced by T cells and is pleiotropic in activity. IL-17 is a 158 amino acid residue polypeptide with a 21 amino acid signal sequence and a mature polypeptide of 137 amino acids. It is a disulfide-linked homodimer. Based on the presence of spatially conserved cysteine residues in the IL-17 family of proteins, there are six family members in humans and mice, IL-17, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F (Aggarwal and Gurney 2002). Like IL-17 itself, several of the family members appear to modulate immune function. Produced by mouse CD4$^+$ T cells, IL-17 induces IL-6, MCP-1, prostaglandin-$E_2$ ($PGE_2$) and granulocyte colony-stimulating factor (G-CSF) by fibroblasts, keratinocytes, epithelial cells, and endothelial cells. It induces ICAM-1 surface expression, proliferation of T cells, and the differentiation of CD34$^+$ marrow progenitors into neutrophils (Fossiez *et al.* 1998). The ubiquitously distributed receptor is a type 1 transmembrane glycoprotein of 830 amino acids in length.

***xiv. Interleukin-18 (IL-18)*** IL-18 is a 24-kDa, nonglycosylated polypeptide that lacks a classical signaling sequence. Its structure resembles IL-1 and the propeptide undergoes proteolytic cleavage by interleukin-1 $\beta$-converting enzyme (ICE) or another caspase to produce an 18-kDa bioactive molecule. There is 64% sequence homology between mouse and human IL-18. IL-18 induces the production of IFN- $\gamma$ by T cells and NK cells and the expression of Fas ligand (FasL) on a variety of all types. IL-18 activates NF-$\kappa\beta$ and the induction of various chemokines. IL-18 plays an important role in the early antibacterial host response (Weijer *et al.* 2003).

***xv. Interleukin-20 (IL-20) and Interleukin-21 (IL-21)*** IL-20 induces keratinocyte differentiation and proliferation. IL-21 is a four-helix-bundle cytokine similar in structure to IL-15 and sharing sequence homology with IL-2 and IL-4. Murine IL-21 is 57% homologous to human IL-21. The IL-21 receptor utilizes the common $\gamma$ -chain. IL-21 is produced by the TH2 cells. The actions of IL-21 are pleiotropic and seen on B cells, T cells, NK cells, and dendritic cells. IL-21 induces apoptosis in resting and activated B cells, an effect counteracted by activation of CD40. It also upregulates production of IgG1 and inhibits IgE, in fact it inhibits many IL-4 activities. IL-21 also inhibits dendritic cell differentiation. IL-21 enhances the activity of activated NK cells and mediates the proliferation and expansion of T-cell subsets. IL-22 induces acute phase reactants by hepatocytes and reduces IL-4 production by TH2 (Mehta *et al.* 2004). Recombinant antigens and antibodies are commercially available for murine IL-20, IL-21, and IL-22.

b. The Transforming Growth Factor-$\beta$ Superfamily (TGF-$\beta$SF) of Cytokines Members of TGF-$\beta$ family share 25–40% sequence homology with TFG-$\beta$1 and a monomeric structure that consists of two antiparallel pairs of $\beta$-strands forming a flat curved surface, a separate long $\alpha$-helix, and a disulfide rich core with a characteristic cysteine knot. Most TGF-$\beta$SF members are disulfide-linked homodimers; however, three members lack the seventh conserved cysteine residue and are not covalent homodimers. Members of the TGF-$\beta$SF include TGF-$\beta$1, TGF-$\beta$2, TGF-$\beta$3, activins, inhibins, bone morphogenic proteins (BMP), growth differentiation factors (GDF), glial-derived neutrophic factors (GDNF), and müllerian inhibiting substance (MIS).

TGF-$\beta$1 is stimulatory for cells of mesenchymal origin and inhibitory for cells of epithelial or neuroectodermal origin. In the murine immune system TGF-$\beta$1 is the mediator of immune suppression via CD4$^+$CD25$^+$Tr cells and, at least in the case of suppressing CD8$^+$ effector T cells, involves TGF-$\beta$ receptor II on these cells (Powrie 2004; von Boehmer 2005). In addition TGF-$\beta$ is known to inhibit B-cell proliferation and it promotes isotype switch to IgA. Oral tolerance to TH2 responses (against

food allergins) is mediated by TGF-β1 (Mucida *et al.* 2005). TGF-β has a wide range of effects on cell growth differentiation and malignant transformation (Letterio 2005). Murine TGF-β1 may be quantified in serum or plasma using commercially available ELISA kits.

Antibodies are also available which specifically bind murine BMP, activin A, activin C, and GDF-1, -3, -5, -8, and -9, although they are not recommended for ELISA development.

c. THE TUMOR NECROSIS FACTOR SUPERFAMILY (TNFSF) TNF-related ligands share many features but high amino acid sequence homology is not one of them. With the exception of nerve growth factor and TNF-β, all ligands are type Ii transmembrane proteins (extracellular C-terminus) that contain a short cytoplasmic segment and a long extracellular region. TNF-β is fully secreted and has a nonfunctional transmembrane segment. TNFSF members form trimeric structures and their monomers are composed of β-strands oriented into a two-sheet structure. Receptors for the TNFSF ligands also belong to a superfamily, TNFRSF (Gruss and Dower 1995), and are characterized as type I transmembrane proteins (with their amino termini outside of the cell), with extracellular cysteine-rich structural motifs. TNFRSF members exist both as membrane and soluble forms. Commercially available ELISA kits or matched antibody for development of assays are available to quantify the murine receptors and ligands of the TNFSF discussed in this section.

***i. Tumor Necrosis Factor-α (TNF-α)*** TNF-α is expressed as a 26-kDa membrane glycoprotein and the soluble glycoprotein is generated by proteolytic cleavage via TNF-α converting enzyme (TACE). The 17-kDa homotrimer cleavage product circulates. Mouse TNF-α has 79% sequence homology with human. TNF-α is expressed widely on tissues throughout the body (Goetz *et al.* 2004). TNF-α is a strong mediator of inflammation and immune function, or regulates on growth and differentiation, and is cytotoxic for many transformed cells.

***ii. Tumor Necrosis Factor-β (TNF-β)*** TNF-β, also known as lymphotoxin-α (LT-α), circulates as a 25-kDa glycosylated polypeptide homotrimer. TNF-β binds to the same receptors as TNF-α. Mouse TNF-β is 72% identical to human. TNF-β does not have a transmembrane form. However, TNF-β can bind to the membrane-anchored LT-β to form a heterotrimer that binds both the LT-β receptor and the TNFRI receptor. T, B, and NK cells make TNF-β, which mediates inflammation and immune function and influences healing.

***iii. CD40L*** CD40L (TNFSF5, CD154) is a 39-kDa, type II, transmembrane glycoprotein that can be proteolytically cleaved to 15- to 18-kDa soluble forms with full biologic activity. It forms natural trimeric structures and the mouse CD40L shares 73% sequence identity with human CD40L. Cells expressing CD40L include B cells, $CD4^+$ and $CD8^+$ T cells, monocytes, NK cells, and γ T cells (Toubi and Shoenfeld 2004). On binding CD40, the complex initiates signals important for cell proliferation or apoptosis. Cross-linkage between T and B cells allows CD40 to transduce the tyrosine kinases Lyn and Syk, and activate phospholipase C, $IP_3$, and DAG. When combined with other cytokines, ligation of B-cell CD40 provides the second signal allowing differentiation of B cells to plasma cells (Goldsby *et al.* 2003).

***iv. CD30L*** CD30L (TNF5F8, CD153) is a 40-kDa glycoprotein with 72% sequence homology between murine and human molecules. CD30L is expressed on monocytes, macrophages, B cells, activated T cells, neutrophils, megakaryocytes, resting $CD2^+$ T cells, erythroid precursors, and eosinophils. Ligation to CD30 can induce either proliferation or apoptosis.

***v. Fas Ligand (FasL)*** FasL (also known as TNFSF6), is a 40-kDa glycoprotein that, after cleavage, forms a 70-kDa homotrimer that is active only in membrane form in the mouse. Polymorphisms in FasL also exist and a single amino acid substitution in position 273 (Phe to Leu) results in the generalized lymphoproliferative disease (gld) mutation. FasL is expressed on cells of the adaptive and innate immune systems, as well as cells of the lung and intestine. There is 77% sequence homology between murine FasL and human FasL (Lynch *et al.* 1994). Ligation of Fas by FasL on mature T cells leads to activation of the caspase cascade and apoptosis. This is a major homeostatic mechanism regulating the size of the T-cell pool and for eliminating T cells that repeatedly encounter self-antigens (Goldsby *et al.* 2003).

***vi. TNF-Related Activation-Induced Cytokines (TRANCE)*** TRANCE, also called RANK ligand and osteoprotegerin ligand (OPGL), is an osteoclast differentiation factor. Mouse and human share 85% sequence homology, and TRANCE is expressed on T cells and T-cell rich organs such as thymus and lymph nodes.

***vii. TNF-Receptor Superfamily (TNFRSF)*** TNFRSF members mediate the cellular effects of TNFSF members. TNFRI and TNFRII bind TNF-α and it appears TNF-β complexes with LT-β and the complex binds to TNFRI or LT-β receptor. CD40 is associated with B-cell proliferation but is expressed on many cells throughout the body. Mouse CD40 shares 62% sequence homology with human CD40; however, the mouse molecule has a 28 amino acid extension of its cytoplasmic tail. CD30 has 480 amino acid residues and a 90 amino acid deletion in the extracellular region compared to human. It is expressed on $CD4^+$ and $CD5^+$ T cells and ligation results in production of IL-5. Murine Fas lacks 8 amino acid residues found in human and shares only 50% sequence homology. Soluble forms result from alternative gene splicing and circulate as dimers or trimers. Fas is expressed by CD34 stem cells, fibroblasts, NK cells, keratinocytes, hepatocytes, B and T cells (and their precursors), and eosinophils. Osteoprotegerin (OPG) inhibits the action of osteoclasts and is a secreted member of the TNFRSF, although it has no transmembrane segment and circulates as a disulfide-linked homodimer. Murine TROY, also named toxicity and JNK inducer (TAJ) and TNFRSF19, shares 92% homology with human in its extracellular domains.

d. INTERFERONS Interferons are a group of related but distinct proteins that share more than 95% amino acid sequence homology. Members of the type I interferon family share a common cell surface receptor composed of two subunits. Commercially available ELISA kits may be used to quantify murine IFN-α, IFN-β, and IFN-γ.

*i. INTERFERON-α (IFN-α)* IFN-α is induced in a wide variety of cells, including monocytes and macrophages, in response to viral infection. One known inducer is double stranded ribonucleic acid (dsRNA). IFN-α (and IFN-β) induce resistance to viral replication by binding the IFN-α/β receptor, which activates the JAK-STAT pathway, inducing several genes. One of those genes is ribonuclease, which degrades viral RNA. Binding of IFN-α to NK cells enhances their lytic activity for virally infected cells. IFN-α is secreted by leukocytes.

*ii. INTERFERON-β (IFN-β)* IFN-β has all the functional characteristics as IFN-α and binds a common receptor. IFN-β is secreted largely by fibroblasts.

*iii. INTERFERON-γ (IFN-γ)* IFN-γ is secreted by TH1 cells, NK cells, and cytotoxic T cells (Tc), which activates macrophages to secrete TNF-α, express class II major histocompatibility complex (MHC) molecules, and produce antimicrobial activities. IFN-γ secretion by TH1 also induces antibody-class switch to IgG2a, which supports phagocytosis and complement fixation. IFN-γ promotes differentiation of Tc from $CD8^+$ precursors that will be involved in the effector response to viral infections and intracellular pathogens. IFN-γ also inhibits the expansion of TH2 cells. IFN-γ secretion is induced by successful stimulation of T cells by antigen presenting cells.

e. CHEMOKINES Chemokines, along with adhesion molecules, are the principle controllers of leukocyte migration and as such directly affect leukocyte retention and relocation during hematopoiesis and at sites of immune defense and inflammatory disease (Moser *et al.* 2004). Chemokine-induced signaling is via G-protein coupled cell surface receptors. Although most chemokines are secreted proteins, two chemokines, CXCL16 and CX3CL1, are membrane bound.

Two primary subfamilies are recognized based on the arrangement of two $NH_2$-terminal cysteine residues that are either located adjacent to each other (CC) or are separated by a single amino acid (CXC). Two minor subfamilies include chemokines with a single cysteine resides (XCL1, XCL2) and a chemokine with three amino acids separating the cysteine residues (CX3CL1). Functionally conserved regions of the N-terminus of each member mediate receptor binding and extracellular matrix fixation (or binding cell surface glycosaminoglycans). Many chemokines are designated with a name given at the time of their identification; all have also been assigned a name based on their structural motif (CC, CXC, XC, CX3C) followed by L for ligand. Receptors are heterotrimers and their activated G-protein subunits stimulate phospholipase Cβ, PIKs, and c-Src tyrosine kinases (Moser *et al.* 2004). Receptors are designated by the type of chemokine they bind (i.e., CXC, CC, XC, or CX3C), followed by R. Mice lack the CXCR1 family of chemokines and receptors found in humans. Table 6-8 lists the murine chemokine receptors and the known ligands. Table 6-3 lists the murine chemokines for which there are either commercial test kits or matched antibodies for kit development.

Two main functional groups define chemokines. Inflammatory chemokines recruit effector leukocytes to sites of infection, inflammation, and repair. Homeostatic chemokines control the navigation of leukocytes during hematopoiesis in the bone marrow and thymus, control homing of cells to spleen, lymph nodes, and Peyer's patches during the adaptive immune response and control immune surveillance in peripheral tissues. Some chemokines participate in both inflammation and homeostasis and are called dual-function chemokines. Many dual function chemokines are highly selective for the recruitment of T cells. Other chemokines have ill-defined functions regarding homeostasis and inflammation but participate in other vital activities such as the role of PF4 (CXCL4) in thrombosis and the role of CXCL10 in gut epithelial cell turnover (Cliffe *et al.* 2005).

Most inflammatory chemokines are thought to be induced and the variety of stimuli that induce their expression is broad. By contrast most homeostatic chemokines are thought to be constitutively expressed. An exception is the inducing effect of lymphotoxin and TNF-α on B-cell attracting chemokine-1 (BCA-1, CXCL13), CCL19, and secondary lymphoid tissue chemokine (SLC, CCL21), which also participate in inflammation.

TABLE 6-8
MURINE CHEMOKINES AND CHEMOKINE RECEPTORS

| Receptor | Chemokines |
|---|---|
| CXCR2 | KC(CXCL1), MIP-2 (CXCL3), LIX(CXCL6) |
| CXCR3 | IP-10(CXCL10), MIG(CXCL9), I-TAC(CXCL11) |
| CXCR4 | SDF-1α (CXCL12) |
| CXCR5 | BCA-1(CXCL13), KS1(BMAC, CXCL14) |
| CXCR6 | CXCL16 |
| Unknown | Lungkine, CXCL15 |
| CCR1 | MIP-1α (CCL3), MIP-1β (CCL4), MIP-1 γ (CCL9), RANTES(CCL5), MCP-5(CCL12), MCP-2(CCL8) |
| CCR2 | MCP-1(CCL2), MCP-2, MARC(MCP-3 or CCL7) |
| CCR3 | Eotaxin(CCL11), Eotaxin-2(CCL24), RANTES, MCP-2 |
| CCR4 | TARC(CCL17), RANTES |
| CCR5 | MIP-1α, MIP-1β, RANTES |
| CCR6 | Exodus-1(CCL6) |
| CCR7 | MIP-3β (CCL19), CCL21 |
| CCR8 | TCA-3(CCL1), Exodus-1, MIP-3α (CCL20) |
| CCR9 | TECK9CCL25) |
| CCR10 | MCP-1, MCP-2, RANTES, CTACK(CCL27), CCL28 |
| CCR11 | MIP-3β |
| $CX_3CR1$ | Factalkine (CX3CL1) |
| Duffy | PF4(CXCL4) |
| XCR1 | Lymphotactin (XCL1) |

Leukocytes also release several kinds of proteases that degrade chemokines at their N-terminus, resulting in the loss of receptor binding, antagonist generation, or enhancing their biologic function. Leukocyte CD26, dipeptidyl peptidase IV is known to act on CXCL9, CXCL10, CXCL11, and CXCL12. Murine sulphostin inhibits the action of CD26 and stimulates G-CSF and granulopoiesis (Abe *et al.* 2005). Matrix metalloproteases (MMPs) are enzymes that degrade extracellular matrix proteins, including stromal cell derived factor-1 (SDF-1, CXCL12) and MCP-1. Table 6-3 lists the murine MMPs that can be quantified by commercially available ELISA kits. In addition to MMPs, cathepsins have been shown to modify chemokines. Table 6-9 lists the murine chemokines and the proteases that degrade them and stimuli that induce them. Certain chemokines may antagonize the activity of other chemokines; for instance, three agonists for the receptor CXCR3 are also antagonists for receptor CCR3 (which is agonized by eotaxin [CCL11]). Because CXCR3 and CCR3 are differentially expressed on TH1 and TH2 cells, these chemokines [monokine induced by IFN-γ (MIG/CXCL9), IFN-inducible protein-10 (IP-10/CXCL10), and IFN-inducible T-cell α chemoattractant (I-TAC/CXCL11)] modulate the TH subpopulation allowed to enter tissue sites favoring TH1 immune polarization (Moser *et al.* 2004). Table 6-10 summarizes some known effects of murine chemokines.

Because chemokines and their receptors are known to modulate many inflammatory diseases including the autoimmune diseases, they have become target for new therapeutics. The chemokine antagonist, Met-RANTES, has been shown to be an effective inhibitor of allergic airway disease in mice. Likewise, deficiencies of chemokines and their receptors in mice have modified disease progression in atherosclerosis, autoimmunity, and also prolong allograft survival (Mackay 2001). In addition, many viral genomes are known to encode structural genes for chemokine antagonists, which appears to be a principal mechanism used by many viruses to evade the host immune system. These present another target for drug intervention for viral infections. Finally, pepducins, derived from the intracellular loops of CXCR1, CXCR2, and CXCR4, specifically inhibit receptor G-protein signaling in mice and prevent fatal sepsis and disseminated intravascular coagulation (Kaneider *et al.* 2005).

TABLE 6-9

MURINE CHEMOKINES, PROTEASE MODIFIERS, AND INDUCERS

| Chemokine | Protease | Inducers |
|---|---|---|
| CCL2 | MMP-1, -3, -8 | H, I, F, IL-4, IL-13, IL-17, FasL, TLR4 |
| CCL3 | Cathepsin-D | H, I, F, IFNα, IFNβ, IL-4, TLR4 |
| CCL4 | Cathepsin-D | H, I, F, TLR4 |
| CCL5 | CD26 | H, TLR4, TLR2 |
| CCL7 | MMP-1, -2, -3 | IFNα, IFNβ |
| CCL8 | MMP-3 | IFN γ |
| CXCL3 | | *Pneumocystis murina* (formerly *P. carinii*) |
| CXCL4 | MMP-9 | |
| CXCL6 | MMP-9 | |
| CXCL9 | CD26 | IFN- γ |
| CXCL10 | CD26 | IFN- γ, L |
| CXCL11 | CD26 | IFN- γ |
| CXCL12 | CD26, MMP-1, -2, -3, -9, -13, -14, Cathepsin-G | |
| CXCL13 | | |

H, histamine; I, immune complexes, F, fibrin, L, lymphotoxin; TLR, toll-like receptor.

Modified from Moser *et al.* 2004.

### 6. Arachidonic Acid Metabolites

Certain growth factors (see Table 6-3) and cytokines activate phospholipases after binding their cell surface receptors. These phospholipases act on membrane phospholipids to release arachidonic acid, a precursor for several eicosanoids. Arachidonic acid is metabolized by any one of three biochemical pathways: the cyclooxygenase (COX) pathway, which forms PGs and thromboxane, the LO pathway, which forms HETEs and leukotrienes (LTs), and the cytochrome P-450 monooxygenase pathway, which forms epoxides and HETEs.

The COX enzymes, COX-1 and COX-2, catalyze the first step in the synthesis of PGs by converting arachidonic acid to prostaglandin $H_2$ ($PGH_2$). $PGH_2$ is chemically unstable and is the precursor for enzymatic and nonenzymatic production of $PGD_2$, $PGE_2$, and $PGF_{2\alpha}$. Thromboxane synthetase converts $PGH_2$ to thromboxane $A_2$ ($TXA_2$) that is quickly converted to thromboxane $B_2$ ($TXB_2$). In vascular tissue, $PGH_2$ is converted to $PGI_2$ or prostacyclin by prostacyclin synthetase (Natarajan and Nadler 2004; Reimers 1999). Although COX-1 is constitutively expressed in most tissues, COX-2 is induced by bacterial lipopolysaccharides, IL-1α, IL-1β, and TNF-α (see the "Cytokines and Chemokines" section).

In the circulation, $PGE_2$ and $PGI_2$ cause vasodilation, whereas $PGF_{2\alpha}$ and $TXB_2$ are potent vasoconstrictors. In the kidney, $PGE_1$, $PGE_2$, $PGD_2$, $PGG_2$, $PGI_2$, and $PGH_2$ produce vasodilation, increased renal blood flow, and urinary excretion of sodium. Renal production is increased by $PGD_2$, $PGE_2$, and $PGI_2$. $PGG_2$, $PGH_2$, and $TXA_2$ modulate platelet aggregation and, following platelet adhesion, they release catecholamines, serotonin, and adenosine diphosphate, which enhance platelet aggregation. $PGI_2$ is a potent inhibitor of platelet aggregation. $PGE_1$ increases, whereas $PGE_2$ decreases, the ability of red cells to pass through capillaries. $PGE_2$ and $PGF_{2\alpha}$ inhibit the activities of T and B cells and the production of ILs and chemokines, which attract monocytes. $PGD_2$ is a potent inducer of chemotaxis for $TH_2$ cells and plays a major role in allergic airway disease. Through the varied activities of vasodilation, vascular permeability, and leukocyte migration, the PGs are potent modulators of inflammation. In addition $PGE_1$ inhibits

**TABLE 6-10**
THE FUNCTION OF MURINE CHEMOKINES

| Receptor | Chemokine | Function | Ref. |
|---|---|---|---|
| CCR1 | CCL3, CCL5 | Antiviral responses | Mackay 2001 |
| CCR2 | CCL2, CCL7, CCL8 | Macrophage migration, especially to brain, IgE class switch | Bot *et al.* 2005<br>Dzenko *et al.* 2005 |
| CCR3 | CCL11, CCL24 | TH2 reactions and eosinophil chemotaxis | Mackay 2001 |
| CCR4 | CCL17 | Skin homing of memory T cells, TH2 migration | Mackay 2001 |
| CCR5 | CCL3, CCL4, CCL5, CXCL11 | Apoptosis of CD1d NKT cells, TH1 responses, and macrophage migration | Mackay 2001 |
| CCR6 | CCL6 | Development of CD4+Tr, and M-cell formation | Mackay 2001<br>Moser *et al.* 2004 |
| CCR7 | CCL19, CCL21 | Maturation of dendritic cells (DC) that are programmed for TH1 induction, migration of skin DC into lymphatics,lymphocyte homing via HEV | Ohl *et al.* 2004<br>Marsland *et al.* 2005<br>Carlsen *et al.* 2005 |
| CCR8 | CCL1 | TH2 responses and recruitment of T and DC to inflamed skin | Gombert *et al.* 2005 |
| CCR8 | CCL20 | Dendritic cell homing | Mackay 2001 |
| CCR9 | CCL25 | Intestinal homing for effector T cells, thymocyte migration | Mackay 2001 |
| CCR10 | CCL27 | Skin homing for memory T cells | Mackay 2001 |
| CXR2 | CXCL1, CXCL3 | Induces the NF-kB signal pathway and controls migration and degranulation of neutrophils | Tsai *et al.* 2000<br>Widney *et al.* 2005<br>Wang *et al.* 2005 |
| CXR2 | CXCL6 | Activates NF-KB and induces proinflammatory cytokines | Chandraseker *et al.* 2003 |
| CXCR3 | CXCL9, CXCL10, CXCL11 | Chemotaxic for lymphocytes and NK cells, controls activated T-cell responses; controls intestinal epithelial cell turnover, T-cell allograft rejection, angiogenesis | Cliffe *et al.* 2005<br>Widney *et al.* 2005<br>Burns *et al.* 2005<br>Sleeman *et al.* 2000 |
| CXCR4 | CXCL12 | Germinal center development, CTL recruitment into tumors, inhibits chemokine-induced myelosuppression, involved with T -and B-cell lymphopoiesis, homing of DC to spleen and migration of sensing neuron progenitors | Cozine *et al.* 2005<br>Broxmeyer *et al.* 2005<br>Belmadani *et al.* 2005<br>Zhang *et al.* 2005 |
| CXCR5 | CXCL14 | B-cell and monocyte chemotaxis, antigen presenting cell development | Moser *et al.* 2004<br>Sleeman *et al.* 2005 |
| CXCR5 | CXCL13 | B-cell follicle interactions, recruitment of B cells, induction of XCL1, XCL2 | Cozine *et al.* 2005 |
| CXCR6 | CXCL16 | DC-T-cell interactions | Mackay 2001 |
| Unknown | Lungkine | Controls neutrophil and T-cell migration into lungs | Rossi *et al.* 1999 |
| CX3CR1 | CX3CL1 | Mediates inflammatory responses, NK migration, access of DC to intestinal lumen | Niess *et al.* 2005<br>Mackay 2001 |
| XCR1 | XCL1, SCL2 | Tumor infiltration by CTL | Huang and Xiang 2004 |
| Duffy | CXCL4 | Induces thrombosis and delays angiogenesis | Yamaguchi *et al.* 2005 |

insulin secretion and release after glucose challenge (see the "Glucose and Carbohydrate Metabolism" section), and it inhibits the lipolytic effects of glucagons, adrenocorticotropic hormone, and epinephrine (see the Lipid Metabolism section) and inhibits the secretion of corticosterone, prolactin (PRL), GH, thyroid stimulating hormone (TSH), and LH. In fact, PGs have a multitude of effects associated with female reproduction (Reimers 1999).

The LOs are classified as 5-LO, 8-LO, 12-LO, and 15-LO based on their ability to insert molecular oxygen at the corresponding carbon position of arachidonic acid. The 5-LO pathway leads to production of 5(S)-HETE and LTs. The 12-LO and 15-LOs form 12(S)- and 15(S)-HETEs in vascular tissue, monocytes, and endothelial cells. The LOs oxidize LDL, inducing growth and inflammatory events in vessels. Likewise HETEs have been implicated in the pathogenesis of atherosclerosis and diabetic vascular disease (see the "Atherosclerosis" section). 12/15-LOs expression in vascular smooth muscle is potentially upregulated by platelet derived growth factor (PDGF) (see Table 6-3) and cytokines IL-1, IL-4, and KC (see the Cytokines and Chemokines section). The 12(S)-HETE increases expression of CS-1 fibronectin on endothelial cells inducing monocyte adhesion (Natarajan and Nadler 2004).

Under the action of 5-LO, arachidonic acid is converted to 5-hydroxyperoxyeicosatetraenoic acid (5-HPETE) and then leukotriene A4 (LTA4), which is unstable. LTA4 is metabolized to LTB4 by LTA4 hydrolase or to cysteinyl leukotrienes (LTC4, LTD4H, and LTC45) by LTC4 synthase. 5-LO expression is limited to neutrophils, eosinophils, monocytes, and mast cells; however, LTA4 hydrolase is expressed in

erythrocytes, T cells, platelets, airway and intestinal epithelial cells, fibroblasts, heart, kidney, and adrenal cortex. Because the latter cells and tissues do not express 5-LO, LTA4 must be delivered to them via myeloid cells by a process known as transcellular biosynthesis (Maclouf 1993). Receptors for LTB4 (BLT1 and BLT2) have been identified and are either widely expressed (BLT2) or are confined to myeloid cells, T cells, and lung cells (BLT1). In addition to attracting myeloid cells to sites of inflammation, LTB4 is a potent inducer of TH1 and TH2 T effector cell chemotaxis (as potent as CXCL12 in the mouse) and $CD8^+$T-effector cell chemotaxis (as potent as RANTES). Like $PGD_2$, LTB4 plays a major role in allergic airway disease (Luster and Tager 2004). Additional information on murine prostanoids and their receptors is available in recent reviews (Jala and Haribabu 2004; Kobayashi and Narumiya 2002). All the arachidonic acid metabolites discussed in this section may be quantified using commercially available ELISA kits (see Table 6-3).

## E. Enzymes

### 1. Alkaline Phosphatase (AP)

AP is an inducible enzyme in which serum activity is increased due to increased synthesis. The exact physiologic function of AP is not known but is thought to transport metabolites across cell membranes. Quantification of serum AP is based on a reaction between AP and a suitable phosphorylated substrate that is susceptible to AP activity.

As in other species, there are two major forms of AP in mice: intestinal AP (IAP) and tissue nonspecific AP (TNAP). Unlike other domestic animal species, IAP activity contributes to serum AP activity, in addition to TNAP. IAP is located along the brush-border of the enterocytes. Intestinal AP activity was shown to vary four-fold between two strains of Swiss mice (Nayudu and Moog 1967). This difference in activity was under polygenic control and influenced by a strain-specific factor in milk. TNAP is found in various tissues, and in each location, post-translation modifications may result in a different isoenzyme. Hoshi *et al.* (1997) used immunohistochemistry to localize these isoenzymes in mice to the following locations: bone tissue (specifically the entire cell surface of preosteoblasts and the basolateral cell membrane of osteoblasts), cartilage (mostly in chondrocytes of the proliferative and hypertrophic zones), the incisors (particularly the cells of the stratum intermedium, the subodontoblastic layer, the proximal portion of secretory ameloblasts, and the basolateral portion of odontoblasts), kidneys (on the brush borders of proximal renal tubules in kidney), liver (on cell membrane of the biliary canaliculi), and the placenta (on trophoblasts).

Serum AP activity can vary due to the type of assay used, age, sex, and strain. Different AP assays vary in the type of substrate used, the pH of the reaction, and the incubation temperature of the reaction, all of which can affect quantitation of AP activity. For example, the Hausamen technique can detect renal and intestinal AP activity, but not hepatic AP activity (Hausamen *et al.* 1967). Loeb *et al.* (1996) and Frith *et al.* (1980) demonstrated that serum AP activity decrease significantly after 3 months of age in BALB/c and C57BL/6 mice of both sexes but increase again in very old (36 month old) mice. The high levels of serum AP seen in juveniles is related to increases in the bone AP isoenzymes, which is associated with osteoblastic activity due to rapid growth. Pickering and Pickering (1984) showed that serum AP activity is lower in males than in females.

Quantification of serum AP measures the total AP activity from all sources, and therefore elevations in AP activity can be a nonspecific determinant of tissue dysfunction, depending on the tissue (and therefore AP isoenzyme) involved. For example, changes in the serum activity of AP due to liver disease only occur if cholestasis is concurrently involved. Mice lacking the gene that modifies TNAP into the bone isoenzyme suffer from skeletal hypomineralization (Anderson *et al.* 2004).

### 2. Alanine Aminotransferase (ALT)

ALT is a cytoplasmic enzyme in which serum activity is increased due to leakage across damaged cytoplasmic membranes. ALT (also known as glutamic pyruvic transaminase [GPT]) reversibly catalyzes the conversion of alanine to pyruvate. Quantification of serum ALT is based on a reaction between ALT and a suitable substrate (such as alanine).

In mice, ALT is found in the highest concentrations in the liver, although activity has also been demonstrated in intestine, kidney, heart, muscle, and brain (Clampitt and Hart 1978). Despite its widespread tissue distribution, ALT is mostly used as an analyte to assess hepatocellular damage (Masaki *et al.* 2005; Taieb *et al.* 2005). An 11,000% increase in serum ALT activity has been reported following infection with mouse hepatitis virus, and significant increases follow infection by *Helicobacter hepaticus* (McCathey *et al.* 1997).

### 3. Aspartate Aminotransferase (AST)

AST is a cytoplasmic and mitochondrial enzyme in which serum activity is increased due to leakage across damaged cytoplasmic and mitochondrial membranes. AST (also known as glutamic oxaloacetate transaminase [GOT]) reversibly catalyzes the conversion of aspartate to oxaloacetate. Quantification of serum AST is based on a reaction between AST and a suitable substrate (such as aspartate).

In mice, AST is found in a variety of tissues, including liver, skeletal muscle, cardiac muscle, erythrocytes, blood vessels, brain, intestine, kidney, lung, testes (Papadimitriou and Van Duijn 1970). The highest specific activity of AST is found in mouse cardiac muscle and lowest in skeletal muscle (Herzfeld

and Knox 1971). In the liver, AST is found mainly in periportal hepatocytes based on histochemical studies (Papadimitriou and Van Duijn 1970). Activity in lung, kidney, intestine, and skeletal muscle is very low when measured by the technique of Bergmeyer and Bernt (1974). Loeb *et al.* (1996) demonstrate significant age-associated increases in serum AST activity in two inbred and two F1 hybrid strains.

Although widely distributed, ALT is mainly used to assess hepatocellular damage, cardiac muscle damage (Naraoka *et al.* 2005; Ray *et al.* 2005), and testicular injury (Santos *et al.* 2005). ALT activity has been used as an indicator of hepatic injury of mice infected with mouse hepatitis virus (Fassati *et al.* 1969).

#### 4. Lactate Dehydrogenase (LDH)

LDH is a cytoplasmic enzyme in which serum activity is increased due to leakage across damaged cytoplasmic membranes. LDH reversibly catalyzes the conversion of pyruvate to lactate. Quantification of serum LDH is based on a reaction between LDH and a suitable substrate (such as pyruvate or lactate).

As in other species, LDH in the mouse is a tetrameric enzyme consisting of either A or B subunits. There are five isoenzymes of LDH (based on differences subunit A and B composition), and these are LDH-1 ($B^4$), LDH-2 ($A^1B^3$), LDH-3 ($A^2B^2$), LDH-4 ($A^3B^1$), and LDH-5 ($A^4$). Specific isoenzyme distribution depends on differential expression of either the A and B subunits (Quimby 1999b). For instance, all embryonic murine tissues contain LDH-5 because the A subunit is only expressed during early fetal development. As the embryo matures, subunit expression can involve both A or B subunits in different tissues, meaning that by birth and sexual maturity, each tissue contain a characteristic LDH subunit profile. In adult mice, the heart and erythrocytes contain LDH-1 and LDH-2, whereas most other tissues have LDH-3. Skeletal muscle and hepatocytes fail to express subunit B and therefore are composed predominantly of LDH-5.

Serum LDH activity can vary due to age and sex. LDH levels have been shown to be higher in males versus females of the BALB/c strain (Frith *et al.* 1980). Serum LDH levels increase with age in BALB/c and C57BL/6 mice of both sexes (Frith *et al.* 1980). Serum LDH can also be elevated falsely by hemolysis.

Quantification of serum LDH measures the total LDH activity from all sources. However, damage to a particular tissue will result in increased activity of that isoenzyme in serum. In general, the highest activity of LDH in the mouse is in skeletal muscle, with decreasing activity in the heart, liver, kidney, and intestine. Serum LDH-5 activity rises within 72 h after inoculating mice with the mouse hepatitis virus (Fassati *et al.* 1969). Mice infected with the LDH virus (LDV) exhibit increased serum concentrations of LDH, isocitric dehydrogenase, malic dehydrogenase, phosphohexase isomerase, and AST (Notkins 1965). Along with AST and CK, LDH is considered an excellent marker for cardiac injury (Naraoka *et al.* 2005; Ray *et al.* 2005).

#### 5. Ornithine Transcarbamylase (OTC)

OTC is a mitochondrial enzyme in which serum activity is increased due to leakage across damaged mitochondrial membranes. OTC is found primarily in the liver of mice, and there increases in serum activity reflect severe injury to hepatocytes. Abnormal OTC activity has been described in mice having the sparse-fur (spf/Y) mutation. They serve as a model for the most common inborn error of urea synthesis in humans. Assays for mouse OTC have been developed to monitor activity levels following gene transfer or liver transplantation (Batshaw *et al.* 1999; Ye *et al.* 2001).

#### 6. Creatine Kinase (CK)

CK is a cytoplasmic and mitochondrial enzyme in which serum activity is increased due to leakage across damaged cytoplasmic and mitochondrial membranes. CK reversibly catalyzes the phosphorylation of adenosine diphosphate (ADP) to ATP, using creatine phosphate as the donor for the phosphate group. Quantification of serum CK is based on a reaction between CK and a suitable substrate (such as creatine phosphate).

As in other species, cytoplasmic CK is a dimeric enzyme consisting of either M or B subunits. There are three isoenzymes of CK (based on differences subunit M and B composition), and these are CK-1 (BB), CK-2 (MB), and CK-3 (MM). Brain contains CK-1, skeletal muscle contains CK-3 (MM), and cardiac muscle contains CK-1, CK-3, and CK-2 (MB) (Quimby 1999b). In the mouse, the greatest CK activity is found in skeletal muscle, with much less activity found in the heart and brain. Mitochondrial CK (CK-Mt) is found in mitochondria of many tissues.

Serum CK activity is affected by age, sex, and method of collection and anesthesia. Patrick *et al.* (1983) found that compared to jugular vein collection, cardiac puncture was associated with lower CK activity. Levels of serum CK activity have been reported for BALC/cAnN mice and C57BL/10 mice of varying ages and sex (Suh *et al.* 1994).

Serum CK activity is a useful and specific marker enzyme of muscle injury, because CK in central nervous tissue does not cross the blood-brain barrier. Suh *et al.* (1994) compared normal C57BL/10 mice with heterozygous male and homozygous female mice carrying the *mdx* (mutant dystrophin) allele, and found that homozygous females have 12- to 15-fold increases and heterozygous males (mdx/Y) have 30 fold increases in plasma CK compared to wild-type mice. Plasma CK levels correlated with skeletal muscle necrosis in these dystrophic mice.

Mice have been engineered that lack cytoplasmic CK and CK-Mt (ter Veld *et al.* 2005). Mitochondria from heart or skeletal muscle from double KOs had higher ADP

concentrations compared to wild-type animals, suggesting the higher concentrations contribute to the control of the reduced cytosolic ATP free energy potentials seen in double KOs.

#### 7. Aldolase

Aldolase is a cytosolic enzyme that can alter its distribution between soluble and particulate forms, according to the metabolic status of the tissue. In adult mice, nine aldolase isoenzymes are known to occur in tissues with significant activities in the muscle, brain, liver, kidney, and spleen. In the mouse liver aldolase, together with fructokinase and triokinase, metabolize fructose (Hagopian *et al.* 2005). Everett and Harrison (1983) report no apparent advantages in mice in the measurement of aldolase over other enzymes known to have specific liver or muscle activity.

#### 8. Sorbitol Dehydrogenase (SDH)

SDH is a cytoplasmic and mitochondrial enzyme in which serum activity is increased due to leakage across damaged cytoplasmic and mitochondrial membranes. SDH (also known as iditol dehydrogenase [IDH]) reversibly catalyzes the conversion of fructose to sorbitol. Quantification of serum SDH is based on a reaction between SDH and a suitable substrate (such as fructose).

SDH is located primarily liver, kidney, and seminal vesicles. The activity of SDH is usually low in the serum and rises during hepatic injury. However, the labile nature of SDH during handling makes is less suitable overall as a indicator of hepatic dysfunction compared to over liver-specific enzymes (such as AST). Mice with the gene for SDH knocked out have been used to study the role of sorbitol accumulation in diabetic albuminuria (Ii *et al.* 2004).

#### 9. Amylase

Amylase is a cytoplasmic enzyme in which serum activity is increased due to leakage across damaged cytoplasmic membranes. Amylase hydrolyzes complex carbohydrates to form maltose and glucose in the presence of free calcium ions. Quantification of serum amylase is based on a reaction between amylase and a suitable substrate (such as starch).

Similar to humans and pigs (but not dogs, cats, cattle, and horses), expression of amylase in mice is related to two distinct but closely linked loci (Meisler *et al.* 1983). Salivary amylase is the gene product of *Amy-1* (salivary), and appears to be a single enzyme. Pancreatic amylase is the gene product of *Amy-2*, and based on electrophoretic studies in inbred mice, there appear to four isoenzyme classes: A1, A2, B1, and B2.

Similar to other domestic animal species, pancreatic amylase is filtered through the glomerulus, but unlike other domestic species, pancreatic amylase is not resorbed by renal tubular epithelial cells and is excreted rapidly in the urine. Therefore normal serum amylase activity in mice consists mainly of salivary amylase (Mackenzie and Messer 1976).

Despite this, elevations in serum amylase activity is usually considered a reliable marker for pancreatitis in mice (Nathan *et al.* 2005). Ross *et al.* (1974) reported two- to three-fold increases in serum amylase activity in mice infected with Coxsackievirus of salivary and pancreatic trophism. Alterations in the activity of specific pancreatic isoenzymes have been shown in streptozotocin-induced diabetes in mice (Quimby 1999b).

#### 10. Other Enzymes

There are two pancreatic lipase isoenzymes in mice. Serum lipase activity has been used to monitor cerulean-induced acute pancreatitis in mice (Cuzzocrea *et al.* 2004). Recently a new colipase-dependent lipase has been described in suckling mice (D'Agostino and Lowe 2004).

The enzyme 5′-nucleotidase was measured in the serum of normal mice using a simple one-step kinetic method (Dooley and Racich 1980). A reference range of $10.9 \pm 4.5$ (SD) U/l has been recorded in 100 mice, and it is thought but not proven to be a good indicator of hepatic injury (Clampitt and Hart 1978).

Glutamate dehydrogenase (GDH) has been measured in the tissues and serum of mice. GDH is known to play a key role in insulin secretion (Carobbio *et al.* 2004). The activity of GDH is fivefold greater in the liver than in the kidney and brain, and the authors speculated that its measurement in serum would be a sensitive indicator of hepatic cell injury. Serum GDH activity is also elevated in mice on caloric restriction (Hagopian *et al.* 2003).

### F. Endocrine Hormones

#### 1. Corticosterone and Adrenocorticotropic Hormone (ACTH)

Corticosterone is the major glucocorticoid secreted by the adrenal cortex of mice. It functions as a regulator of carbohydrate, protein, and fat metabolism and modifies the host response to stress. The male mouse has a well-defined diurnal concentration pattern, with a maximum concentration of 9 μg/dl at the start of the dark cycle and a minimum concentration of 5 μg/dl shortly before the end of the dark cycle (Ottenweller *et al.* 1979). In contrast, female mice have a minimum concentration of 13.5 μg/dl at the beginning of the dark cycle and a maximum of 40 μg/dl well into the dark period (Scheving *et al.* 1983). The length of the dark cycle was different in each study. It may be measured using radioimmunoassay, ELISA, or fluorometric assay in mouse serum or plasma.

Corticosterone circulates in both free and protein bound forms. In the mouse, greater than 99% circulates bound to cortisol-binding globulin (CBG) and albumin. The diurnal variation in corticosterone levels is paralleled by the diurnal pattern of CBG. Urinary and salivary corticosterone is derived only from the free plasma fraction. Corticosterone synthesis

and secretion may be influenced by many drugs (Woodman 1997). Measurements of corticosterone in unrestrained mice using indwelling catheters have elucidated the necessity of eliminating stress for accurate interpretation of data (MacLeod and Shapiro 1988). Both handling and crowding laboratory mice can cause elevations in corticosterone (Balcombe *et al.* 2004; Fullwood *et al.* 1998).

C57BL/6 *ob/ob* mice have elevated corticosterone levels that increase markedly after 40 weeks of age, preceding elevations in serum glucose (Garthwaite *et al.* 1980). Lean C57BL/6 controls had lower serum concentrations of corticosterone that declined between 5 and 20 weeks of age. Food restriction (to 60% of ad libitum) profoundly affects the diurnal increase in plasma corticosterone in mice. At 20:00 hours (8 P.M.), the daily maximum in this study, food restricted BALB/c mice had 300% more corticosterone compared to controls. These mice also had significantly reduced thymic weight and inflammation in response to the injection of carrageenan subcutaneously. The authors report that the magnitude of carrageenan-induced inflammation fluctuates with a diurnal trough, which coincides with peak corticosterone levels (Klebanov *et al.* 1995).

Corticosterone is synthesized in response to ACTH, which is made in the anterior pituitary. ACTH release is episodic (not at fixed intervals) and does not involve steroid feedback. ACTH concentration peaks in the early evening in mice and can be reversed by reversing the light cycle. ACTH is highly conserved with the mouse ACTH differing from human by only two amino acid residues. RIA may be used to measure ACTH in the mouse (daily levels vary from 2.6–5.4 ng/ml) and to demonstrate rhythmic cycling. Samples must be collected at 5- to 30-minute intervals. Commercial ELISA kits for ACTH are also available (Quimby 1999b)

KO mice incapable of synthesizing corticotrophin releasing hormone or ACTH may require corticosterone supplementation in drinking water and are particularly susceptible to hypoglycemia during fasting. Fetuses of homozygous KO CRH null mothers must be supplemented from gestational day 12 to weaning with 30 mg/ml corticosterone in drinking water (Mugila *et al.* 1995). Oxytocin gene KO mice respond to a psychogenic stressor with more anxiety-related behavior and more corticosterone production (Amico *et al.* 2004).

### 2. Luteinizing Hormone (LH)

LH promotes follicular development, increases estradiol secretion in the preovulatory follicle, causes follicular rupture, converts the preovulatory follicle into a corpus luteum, increases progesterone production by the corpus luteum, and, in the male, increases production of testosterone from Leydig cells (Woodman 1997). LH is a glycoprotein composed of α- and β-chains. The amino acid sequence of the α-chain is identical to that of follicle-stimulating hormone (FSH); however, the β-chain is specific and confers the receptor binding properties on the hormone. A homologous RIA for rat LH using antisera to rat LH and purified rat LH has been employed to measure LH in mice (Quimby 1999b). LH and follicle-stimulating hormone (FSH) are secreted by the same anterior pituitary cell in response to stimulation by gonadotrophin-releasing hormone (GnRH), which is synthesized in the hypothalamus. GnRH is identical in mouse and humans, and its differential stimulatory effect on gonadotropes, producing FSH or LH is controlled through GnRH pulse frequency.

Pulsatile secretion of LH leads to great variations in blood concentration. In female mice, there is a 10- to 25-fold increase in basal LH levels in the afternoon of proestrus. In a study comparing young cycling C57BL/6 female mice to old females (40% of which not cycling), Flurkey *et al.* (1982) found lower LH levels in the older group and a more rapid rise in LH among younger cycling mice. These authors found that reproductive failure in male mice correlated with the loss of episodic release of LH. Female homozygous KO mice lacking the gene for the immediate early transcription factor NGFI-A were infertile secondary to LH-β deficiency. Although ovaries from these mice had a similar number of follicles, they lacked corpora lutea (Lee *et al.* 1996). Basal levels of serum progesterone were lower in NGFI-A females (6.3 ± 2.4 ng/ml) compared to wild type (11.3 ± 3.6 ng/ml), whereas serum estradiol levels were similar in deficient (50.7 ± 36.9 pg/ml) and wild-type (52.0 ± 34.3 pg/ml) mice. Decreased amounts of mRNA encoding LH-β, but not FSH-β, were observed in NGFI-A-deficient mice; no changes were observed in mRNA levels for PRL or GnRH receptor between deficient and wild-type mice. An ovariectomy normally removes gonadal feedback inhibition, allowing for increased amounts of LH-β and FSH-β in the pituitary; however, ovariectomy of NGFI-A deficient female mice lead to an increase in FSH-β only. Homozygous NGFI-A deficient males did make lower amounts of LH-β compared to wild-type males, but they made significantly more LH-β than did NGFI-A–deficient females. The levels of LH in males appeared to be sufficient for fertility, and they had normal serum testosterone levels. These results suggest that NGFI-A acts synergistically with transcription factor SF-1 to regulate the promoter for LH-β gene expression. The simultaneous activation of both SF-1 and NGFI-A by GnRH appears to confer the specificity of LH-β synthesis. Female transgenic mice overexpressing LH are anovulatory, develop granulosa cell tumors, and undergo precocious puberty (Mann *et al.* 2003). Excellent reviews detailing the use of transgenic and KO mice in elucidating the secretion and function of LH have been published (Burns and Matzuk 2002; Huhtaniemi *et al.* 2002; Wells and Murphy 2003).

### 3. Follicle-Stimulating Hormone (FSH)

FSH stimulates the growth and maturation of ovarian follicles in the female and promotes the latter stages of spermatogenesis in males. It acts principally on the Sertoli cells of the seminiferous tubules of the testis and induces the secretion of androgen-binding protein and inhibin. In females, FSH and

LH have distinct secretory patterns that are synchronized to the estrous cycle; mouse estrous cycles have a 4- to 5-day periodicity. In addition to estrous cycle dependent rhythms, both LH and FSH have ultradian pulses and show circadian periodicity, with highest levels occurring during the dark period of the light cycle. Testosterone, estrogen, and progesterone all provide feedback regulation of LH and FSH secretion (Woodman 1997). Because rat and mice share considerable sequence homology for the FSH-β chain, the rat RIA kit from National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) can be used to measure FSH in the mouse (Dalterio *et al.* 1981). A mouse specific RIA is commercially available.

In cycling female mice, the plasma levels of FSH increase from 80 to 120 ng/ml during proestrus and 250–300 ng/ml during estrus. Although Bronson and Desjardins (1977) found age associated decreases in serum LH and testosterone in male $CBF_1$ mice experiencing decreased sperm production, no similar decreases were observed in FSH concentration. No significant differences in the twofold increased FSH levels were seen among young versus old males following castration. This appears to differ from results obtained from young versus old rats (Finch *et al.* 1977). The regulation and function of FSH has been studied in transgenic and KO mice (Cooke and Saunders 2002; Wells and Murphy 2003).

#### 4. Androgens

Testosterone promotes spermatogenesis and provides feedback regulation of gonadotrophin synthesis. Through its action on the epididymis, proteins necessary for spermatozoal maturation are synthesized. Dihydrotestosterone (DHT) promotes the growth and differentiation of accessory sex organs (DePaolo and Masoro 1989). In addition to their effects on reproductive organs, testosterone and DHT have physiologic functions on the central nervous system, cardiac tissue, and the liver. In the mouse, testosterone stimulates the growth of kidneys and synthesis of erythropoietin (Woodman 1997). LH stimulates synthesis of testosterone from cholesterol by the interstitial Leydig cells of the testis. In target tissues, the enzyme 5α-reductase converts testosterone to DHT. In the fetal testes, Leydig cells first become identifiable during the rise of testosterone. In most mammalian species, Leydig cells disappear when testosterone levels fall during gestation. However, the mouse is an exception and Leydig cells do not undergo regression postnatally (Aoki 1970).

Testosterone and DHT circulate in both free and protein bound forms. Ninety-eight percent of total testosterone is bound to protein, mainly albumin. Mice, unlike humans, dogs, monkeys, and cats, do not have circulating sex hormone-binding globulin. In mice, a pulse release pattern may be seen within their diurnal rhythm of testosterone. Testosterone can be measured in mice using RIA or ELISA.

Plasma testosterone was not found to change during the average lifespan of C57BL/6 and DBA/2J mice (Finch *et al.* 1977). This is in contrast to CBF1 mice, Wistar and Long-Evans rats, and humans, which experience greater than a 30% decrease in testosterone at midlife (Bronson and Desjardins 1977). In contrast to testosterone levels in older mice, castration-induced elevation of LH was impaired in 28-month-old C57BL/6J mice compared to 12-month-old mice. Androgen receptor KO mice have been described (Cooke and Saunders 2002).

#### 5. Estradiol ($E_2$)

The granulosa cells of the mature Graafian follicle are the main source of 17β-estradiol ($E_2$) in mammals. FSH stimulates the activation of aromatase in follicles, which is responsible for the conversion of androgens to $E_2$. $E_2$ is also synthesized by the testis, adrenal, liver, and skin, although in much lower amounts than by the ovary. $E_2$ provides negative feedback control over LH secretion, and it stimulates PRL secretion in mice. $E_2$ promotes the growth and development of the female reproductive tract, external genitalia, and the ductal system of the mammary glands. In addition, $E_2$ sensitizes the follicle to FSH. $E_2$ is measured in mice using RIA and ELISA.

High circulating levels of $E_2$ precede the preovulatory surge in LH. Ryan and Schwartz (1980) reported basal levels of $E_2$ in mice of 1 to 5 pg/ml. Holinka *et al.* (1978) studied circulating levels of progesterone and estradiol in young and old C57BL/6 female mice during gestation. They found that the old multiparous females have delayed and reduced preparturitional rise is plasma $E_2$ compared to young females. Because preparturitional $E_2$ is thought to regulate uterine progesterone levels, the decline in $E_2$ seen in older females coupled with elevated progesterone may delay the onset of labor and lead to prolonged gestation. When sex steroid hormones such as $E_2$ bind their receptor, they induce a conformational change that allows the complex to bind DNA response elements on nuclear target genes and associate with coactivators and transcription factors to form an active transcriptional complex. This complex is responsible for initiating the transcription of the target gene.

One coactivator that associates with the active transcriptional complex involving sex hormones is steroid receptor coactivator-1 (SRC-1). To better understand its physiologic role during sex hormone–receptor binding, KO mice that were deficient in SRC-1 were created (Xu *et al.* 1998). Deficient male mice had a 34% reduction in testosterone-stimulated prostate growth and small testes compared to wild-type mice. Deficient females had a half-normal uterine growth response to exogenous $E_2$ and only a partial ductal growth response (in the mammary gland) following exogenous $E_2$ and progesterone. Serum concentrations of $E_2$ and testosterone were elevated 1.2 to 1.5 times wild-type levels, indicating an abnormality in the endocrine feedback control system. Although SRC-1 was shown to clearly be necessary for optimal sex hormone-induced cellular activation, the lesion created in this KO mouse was not nearly as profound as that seen in mice with disrupted $E_2$ receptors (Korach *et al.* 1996). Mice with the genes for the $E_2$ receptors and aromatase knocked out have been described (Simpson *et al.* 2005).

#### 6. Progesterone

Synthesized by the corpus luteum before implantation and by both corpus luteum and placenta following implantation, progesterone is necessary for preparing the uterus for implantation and maintaining pregnancy. In addition, a small amount of progesterone is synthesized by the adrenal gland. Activation of the corpus luteum to secrete progesterone requires both LH and PRL. Progesterone provides negative feedback control over LH. In mice, progesterone is measured using RIA or ELISA.

In cycling mice, the levels of progesterone increased from 5 to 35 ng/ml on proestrus and returned back to baseline on estrus. Flurkey *et al.* (1982) compared the plasma levels of LH, progesterone, and PRL in cycling young (10-week) and old (8-month) female C57BL/6J mice. They showed age-related deficits in the preovulatory levels of all three hormones. The LH elevations during the ascending and descending portions of the preovulatory surge were smaller; the slower rises in LH during the ascending phase of the surge correlated with decreased progesterone in older females. There was no correlation between LH or progesterone level and length of estrus cycle.

Holinka *et al.* (1978) observed changes in plasma progesterone levels in young and old female mice during gestation. Older mothers had a much slower decline in circulating progesterone than younger mothers between gestational days 17–23. Because a major decrease in plasma progesterone is thought to be essential for the onset of uterine contractions and parturition in mice, the authors hypothesized that the attenuated decline in older mothers may account for their prolonged gestation.

Surgimoto *et al.* (1997) engineered mice lacking the $PGF_{2\alpha}$-receptor (FP) and found that deficient female mice had normal estrous cycles, ovulation, fertilization, and implantation but did not undergo parturition. These pregnant females were characterized by a decline in preparturition progesterone levels due to delayed luteolysis and failure to develop labor. Close examination revealed that near term there was no expression of the oxytocin receptor in the uterus of FP-deficient mice. When the ovaries of FP-deficient pregnant females were removed on day 19 of pregnancy, progesterone levels fell, uterine oxytocin receptors were expressed, and pups were born alive within 24 h. This study demonstrated that the role of $PGF_{2\alpha}$ is to initiate luteolysis, resulting in an immediate decline in progesterone levels. The subsequent induction of oxytocin receptors and their activation by bound oxytocin initiate labor. It is feasible that the age-associated prolongation of gestation in old female mice may involve a defect in $PGF_{2\alpha}$-induced luteolysis. This mechanism for the induction of labor also explains the well-known observation that aspirin-like drugs (that inhibit COX metabolism) may delay parturition in women.

#### 7. Prolactin (PRL)

PRL, a 23-kDa single-chain polypeptide, is principally made by the pituitary gland but may be also be found in brain, thymus, spleen, lymph nodes, mammary gland, myometrium, sweat gland, lacrimal gland, and bone marrow. More than 300 functions have been attributed to PRL, involving reproduction and lactation, growth and development, endocrinology and metabolism, brain and behavior, immunomodulation, and electrolyte balance. It mediates effects by endocrine, paracrine, and autocrine mechanisms. In mice, PRL is of major importance for maintenance of the structure, life span, and function of the corpora lutea and the development and maintenance of the mammary gland and lactation. However, PRL-receptor KO males have no major defect in fertility. PRL plays a role in anxiety-related behaviors, bone development, and abdominal fat deposition in both sexes (Kelly *et al.* 2001). Its role in immune function is more controversial, although it appears to modulate immunity during times of stress (Dorshkind and Horseman 2000).

PRL mediates its effects by binding the PRL receptor (PRLR). Mice have two major forms of PRLR that are created via alternative splicing of a single gene. The PRLRs are members of the class I cytokine receptor superfamily. Although most functions of PRL are mediated by the unmodified 23-kDa peptide, post-translational modification allows variants of PRL to bind its receptor eliciting transcription of genes necessary for tissue specific changes (Harris *et al.* 2004).

PRL secretion is under inhibitory control by dopamine and among its secretagogues are thyrotropin-releasing hormone (TRH), vasoactive intestinal peptide, gastrin, serotonin, β-endorphin, oxytocin, angiotensin II, GnRH, and arginine vasopressin. PRL in mice is quantified using RIA using the NIH RP-1 reference standard. Male mice measured during the light phase have <1 ng/ml whereas the range during the dark phase was 10–20 ng/ml (DePaolo and Masoro 989).

#### 8. Oxytocin

Oxytocin, a 21-kDa nonapeptide, is synthesized as a prohormone in neurons whose bodies are located in the supraoptic and paraventricular nuclei at the base of the hypothalamus. Once synthesized, the prohormone is packaged into neurosecretory granules and transported down the axons of these neurons transport synthesized oxytocin to the pars nervosa of the pituitary gland. During transport, the molecule is cleaved to yield the 10-kDa carrier proteins, neurophysin I, and the 11-kDa oxytocin. The primary stimulus for oxytocin release is mechanical stimulation of the mammary gland and distention of the reproductive tract (Reimers 1999). There is a single oxytocin receptor (OTR). Investigations using KO mice have shown that oxytocin is required for milk let down by the mammary gland as well as for postpartum alveolar proliferation. In males, oxytocin is required for normal spermiation and sperm transfer. In both genders, oxytocin helps control normal blood pressure and salt intake. KOs display reduced aggression and a striking deficit in the ability to recognize a previously encountered mouse (Young and Gainer 2003). Oxytocin may be quantified in mice

using commercially available ELISA kits or by species-specific RIA. Values in one recent publication showed plasma oxytocin at $1.5 \pm 0.6$ pg/ml in male C57BL/6 mice (Chen *et al.* 2004).

### 9. Vasopressin (AVP)

AVP is synthesized and released by the same neurons previously described for oxytocin, although the two secretory granules rarely colocalize in the same neuron. The carrier protein for AVP is neurophysin II. AVP is released from neuron secretory granules by electrical signals from osmoreceptors measuring the osmolarity of extracellular fluid. Action potentials, generated in the receptors, cause calcium influx into axon terminals and exocytosis of AVP. AVP is transported in the blood to the kidneys, where it binds receptors in the distal segment of the nephron and collecting ducts leading to increased resorption of water (Reimers 1999). There are three AVP receptors, V1aR, VlbR, and V2R; and these are all G-protein coupled receptors. Nonsense mutations of the AVP gene (as seen in the Brattleboro rat) or KD mice develop neurohypophysial (central) diabetes insipidus. Mice with the V2R knocked out develop nephrogenic diabetes insipidus characterized by polyuria, polydipsia, and failure to concentrate urine. When the V1aR gene is knocked out, mice develop an enhanced proliferation of splenic B cells and enhanced IgG1 and IgG2b production to thymic-dependent antigens. When V1bR is knocked out the mice display a marked reduction in social aggression and a deficit in social recognition (Young and Gainer 2003). AVP can be measured by RIA or ELISA and the reported values in normal male C57BL/6 mice are $5.1 \pm 1.5$ pg/ml in plasma (Chen *et al.* 2004).

### 10. Thyroid-Stimulating Hormone (TSH)

TSH is a 28-kDa glycoprotein made by the anterior pituitary gland on stimulation by thyrotropin-releasing hormone (TRH). TRH is made in the hypothalamus. TSH is secreted in a pulsatile manner, similar to ACTH.

TSH secretion is regulated by triiodothyronine ($T_3$), which acts on the hypothalamus to inhibit secretion of TRH and acts on the pituitary gland to inhibit TSH synthesis (Reimers 1999). TSH plays a role in stimulating the thyroid gland to produce thyroxine and it influences the outcome of T-cell development in thymus and intestine. In addition to the thyroid gland, TSH receptors are found on many different populations of hematopoietic cells in bone marrow, subsets of dendritic cells, monocytes, and lymphocytes in the spleen and lymph nodes (Klein 2003; Scofield *et al.* 2005).

KO mice have been created in which genes for TRH (Wells and Murphy 2003; Yamada *et al.* 2003) and TSH receptors (Biesiada *et al.* 1996) have been deleted. In addition, spontaneous mutations in the *Pit1* gene lead to deficiencies of GH, PRL, and TSH (Yeap and Leedman 1999). TSH is measured by RIA and reference ranges established around a 3.1 U/mg reference standard were 1–90 ng/ml (DePaolo and Masoro 1989).

### 11. Thyroxine ($T_4$) and Triiodothyronine ($T_3$)

$T_4$ is synthesized entirely in the thyroid gland, whereas $T_3$ is produced primarily by conversion of $T_4$ to $T_3$ in extrathyroidal tissues. In the thyroid, thyroglobulin is synthesized and stored, and later hydrolyzed to form $T_4$. The tyrosine residues, held in peptide linkage within thyroglobulin, are first iodinated and later iodotyrosines are chemically coupled to form hormonally active $T_4$ and $T_3$. Production of $T_4$ depends on adequate supplies of iodine.

Both $T_3$ and $T_4$ circulate in the blood primarily bound to albumin and $\alpha$-globulin; however, mouse transthyretin does not bind $T_4$. Approximately 85% of $T_3$, the active thyroid hormone, in blood is made through the monodeiodination of the outer ring of $T_4$ in a variety of tissues. Certain drugs, food deprivation, and illness can all effect monodeiodinase activity (Reimers 1999).

$T_3$ actions are mediated via two $T_3$ receptors, TR$\alpha$ and TR$\beta$, which act as hormone-inducible transcription factors and belong to a large superfamily of nuclear hormone receptors including the steroid hormone, vitamin D, retinoic acid, and peroxisomal proliferator receptors (Yen 2003). In mouse and human TR$\alpha$ and TR$\beta$ encode nine mRNA isoforms through alternative splicing; four isoforms TR$\alpha$1, TR$\alpha$2, TR$\beta$1, and TR$\beta$2, are expressed as proteins. TR$\alpha^{o/o}$ KO mice, which lack all products from the TR$\alpha$ locus, are fertile and have normal basal thyroid status but have increased sensitivity to thyroid hormones in the pituitary and liver following provocative testing with increasing doses of $T_3$. TR$\alpha^{-/-}$ have a disruption of the first coding exon in the TR$\alpha$ locus, which prevents transcription of TR$\alpha$1 and TR$\alpha$2 mRNAs but not TR$\Delta\alpha$1 or TR$\Delta\alpha$2. These pups die shortly after weaning unless supplemented with $T_3$ for the first 2.5 months of life, after which they develop normal thyroxine levels. However, both genders are infertile. TR$\alpha2^{-/-}$ overexpresses TR$\alpha$1 and are hypothyroid but have inappropriately normal TSH levels. They exhibit some signs of hyperthyroidism, such as increased heart rate, weight loss, and elevated body temperature. TR$\beta^{-/-}$ lack all TR$\beta$ isoforms and display resistance to thyroid hormones demonstrating the key role of TR$\beta$ in set-point control of the pituitary-thyroid feedback axis. TR$\beta2^{-/-}$ KO mice have resistance to thyroid hormones and elevated $T_3$, $T_4$, and TSH. They have defective TSH suppression by $T_3$. Several double KOs (both TR$\alpha$ and TR$\beta$) have been developed with profound resistance to $T_3$. These targeted mutants have helped to elucidate the full function of thyroid hormones involving: bone formation and mineralization, abnormal development of skeletal muscle, disrupted development of the cones in the retina, abnormalities in the auditory system, cochlear and vestibular structures, delayed small intestine development, impaired thermogenesis, and altered development of the central nervous system and immune system (O'Shea and Williams 2002).

Both total $T_4$ and $T_3$ can be measured by RIA in mice, and reference ranges vary greatly by strain and age. Although $T_3$ is the active hormone, its serum levels are so low that it is a less

reliable indicator of thyroid status. Total $T_4$ ranges in Swiss Webster and ICR mice are 5.5 ± 0.7 and 4.7 ± 0.3 μg/ml, respectively. Total $T_3$ levels range from 65–85 ng/100 ml in both SW and ICR stocks (DePaolo and Masoro 1989). Many factors, including autoantibodies, are known to interfere with thyroid hormone assays (Despres and Grant 1998; Reimers 1999).

#### 12. Parathyroid Hormone (PTH)

PTH is synthesized by the chief cells of the parathyroid gland and secreted as an 84 amino acid peptide. Circulating levels of ionized calcium induce synthesis of PTH. Low calcium stimulates PTH synthesis, and high calcium inhibits secretion. The primary function of PTH is to control calcium concentrations in extracellular fluid and prevent hypocalcemia by increasing calcium resorption from bone, glomerular filtrate, and intestines (Reimers 1999). PTH mediates its effect via its G-protein coupled receptor, PTH1R. Interestingly a second protein, PTH related protein (PTHrP), which is secreted by a variety of tissues and acts by autocrine and paracrine signaling, uses the same PTH1R. PTHrP modulates a wide range of physiologic and developmental responses (Goltzman and White 2000). Mice with targeted mutations of the PTH, PTHrP, and PTH1R genes have demonstrated the critical role of these proteins in regulating both the switch between proliferation and differentiation of chondrocytes and their replacement by bone cells (Schipani and Provost 2003). Serum levels of PTH may be quantified in mice using a commercially available ELISA kit, which can also be used to quantify the protein in rats.

#### 13. Other Hormones

ELISA kits are commercially available to quantify many additional hormones in the serum of mice, including insulin, leptin, GH, epinephrine, orexin A, orexin B, adiponectin, adipsin, and resistin. Each of these hormones have been briefly discussed in the "Glucose and Carbohydrate Metabolism" section.

### G. Liver Function Tests

The liver has many complex functions including protein metabolism, carbohydrate metabolism, and lipid metabolism. The liver is involved with the synthesis of many plasma proteins (including albumin), the conversion of ammonia to urea, and the production of glucose from glucogenic amino acids). The liver is implicated with the regulation of blood glucose levels (via glycogenolysis and gluconeogenesis, as discussed in the "Glucose and Carbohydrate Metabolism" section), the removal of glucose from the blood via GLUT-1 and GLUT-2 membrane transporters, and storage in the form of glycogen. The liver is also involved in cholesterol and triglyceride synthesis, the formation of lipoproteins (discussed in the "Lipid Metabolism" section), and the synthesis of carbohydrates from fats. There are also other more specific functions that the liver facilitates, including the synthesis of heme, the synthesis of many coenzymes, detoxification/biotransformation of exogenous and endogenous substances via the cytochrome P-450 microsomal enzymes, and the synthesis of bile. The role of the liver in these functions for various animals has been reviewed (Tennant 1999; Woodman 1988).

#### 1. Enzymes

The elevation of plasma or serum enzymes usually confined to the cytosol or mitochondria of hepatocytes is helpful in elucidating liver injury. Many factors influence the duration of elevated serum enzyme levels including molecular size, intracellular location, rate of plasma clearance, rate of enzyme inactivation, and hepatic production. Hepatic necrosis is associated with elevations of ALT, AST, SD, and OTC in mice, and extrahepatic cholestasis is associated with elevated AP (Tennant 1999). Please refer to the "Enzymes" section for a description of each enzyme.

#### 2. Bilirubin

Bilirubin is a pigment that is produced by the degradation of the hemoglobin by cells of the mononuclear phagocyte system. There are two main types of bilirubin. Unconjugated bilirubin is a non–water soluble molecule that is transported in blood bound to albumin. Hepatocytes uptake unconjugated bilirubin, where it undergoes glucuronidation to produce the water-soluble form, conjugated bilirubin. Conjugated bilirubin is then excreted via the hepatobiliary system and excreted in bile.

Quantification of serum bilirubin levels is based on the van den Bergh or diazo reaction. Diazo reacts directly with conjugated bilirubin, and with unconjugated bilirubin only after addition of alcohol to the reaction. Therefore, measuring serum bilirubin involves the following steps. First, the level of conjugated bilirubin is measured. Second, alcohol is added to the reactants, which allows quantification of total bilirubin levels. Finally, the level of unconjugated bilirubin is determined by subtracting the conjugated measurement from the total measurement.

The excretory capacity of the liver may be assessed by measuring serum bilirubin. Elevated levels of unconjugated bilirubin are usually observed in situations of increased erythrocyte breakdown, such as in hemolytic diseases. Unconjugated hyperbilirubinemia is also seen in disease in which hepatic uptake, conjugation, and secretion of bilirubin are diminished. Increased levels of conjugated bilirubin are usually associated with intrahepatic cholestasis or extrahepatic bile duct obstruction. Conjugated bilirubin in blood is normally filtered by the glomerulus in small amounts. Conjugated hyperbilirubinemia may result in bilirubinuria (Tennant 1999).

Bilirubin clearance from blood and the role of the constitutive androstane receptor in this process has been studied in normal

and transgenic mice (Huang *et al.* 2003; Huang *et al.* 2004). Studies documenting the bilirubin-metabolism/detoxifying enzymes, their regulatory nuclear receptors, and lipid transporters in mice have been reported (Wagner *et al.* 2005).

#### 3. Bile Acids

Bile acids are cholesterol breakdown products that are secreted by hepatocytes into the hepatobiliary system, and ultimately into the intestinal tract. Bile acids aid digestion by emulsifying dietary lipid aggregates, and solubilizing and transporting lipids in an aqueous environment (in particular fat-soluble vitamins). In the liver, bile acids also play a role regulating the secretion of apolipoprotein B (Elzinga *et al.* 2003).

Extensive enterohepatic recirculation results in almost 99% return of bile acids secreted by the liver from the intestinal tract. Absorption occurs mainly in the ileum under the influence of the apical $Na^+$-dependent bile acid transporter on epithelial cells (Kida *et al.* 2003). Some absorption also takes place in the large intestine. Absorbed conjugated bile acids (from ileum) pass unaltered to the portal circulation where they are removed by $Na^+$-taurocholate cotransporting polypeptide located on the basolateral hepatocyte membrane (Jung *et al.* 2004).

There are two types of bile acids, cholic acid and chenodeoxycholic acid. In mice, they are conjugated with taurine before secretion by the liver. The liver-specific enzymes, bile acid CoA ligase and bile acid-CoA:amino acid N-acyltransferase, are responsible for conjugation (Inoue *et al.* 2004). Stedman *et al.* (2004) described normal serum bile acid levels in normal and transgenic mice.

Elevated serum bile acid levels in mice is usually due to decreased bile acid recycling by the liver, and mainly includes diseases associated with decreased hepatic functional mass and cholestasis (Tennant 1999). Hoda and Green (2003) measured increased levels of serum bile acids after bile duct ligation.

#### 4. Serum Proteins

Albumin is a nonglycosylated protein and is the most abundant protein in plasma. It serves as the most important determinant of plasma oncotic pressure, and it is a major transport protein for both endogenous metabolites and xenobiotics. It is made exclusively by the liver. Serum albumin may be measured by radial immunodiffusion, dye binding reactions (using bromcresol green or bromcresol purple), electrophoresis, and ELISA. Serum albumin levels decrease with age in many inbred strains (Quimby 1999b). Hypoalbuminemia is usually reflective of decreased hepatic synthesis (due to hepatic disease, inflammation, and malabsorptive/maldigestive diseases), increased loss due to hemorrhage or via the intestinal tract (protein-losing enteropathies) and kidneys (protein-losing nephropathies) (Tennant 1999).

Most of the proteins associated with coagulation are synthesized by the liver (except factor VIII), and measurement of fibrinogen or prothrombin time has served as a marker for decreased synthesis due to hepatic injury (Tennant 1999). Prothrombin time will also be increased due to consumption of clotting components and in vitamin K deficiency. Fibrinogen is also an acute phase reactant, and plasma elevations are seen during inflammation (Tennant 1999). Fibrinogen may be quantified in mouse plasma by ELISA.

Ceruloplasmin is copper-containing acute phase reactant and iron transport protein made exclusively in hepatocytes of mice. Its serum levels may be decreased during hepatic injury and increased in response to inflammatory stimuli (Min *et al.* 1991; Shim and Harris 2003).

Many of the complement components are also made in the mouse liver, especially C2, C3, C4, and factor B. Decreased circulating levels may be seen due to hepatic injury or due to consumption during activation. Please refer to the "Complement" section for further details on complement function and measurement.

#### 5. Dye Excretion

Two dyes, sulfobromophthalein and indocyanine green, have been used to assess hepatocellular or bile tract function. Following an intravenous bolus, these dyes are rapidly cleared from the plasma by hepatocytes and excreted into the bile. Both dyes have been used to assess liver function in mice (Hurwitz *et al.* 1994; Huang and Vore 2001).

### H. Kidney Function Tests

The kidney plays a complex role in maintaining homeostasis in the body and is involved in such functions as water and electrolyte balance, nutrient conservation, maintenance of blood pH, and removal of the end products of nitrogen metabolism (such as urea, creatinine, and allantoin). In addition, the kidney produces and responds to a variety of hormones. However, assessing renal function is complicated by the large functional reserve of the kidneys. For instance, increases in urea nitrogen do not occur until 70–75% of renal mass has become functionally compromised. Additionally, assessment of analytes in urine is difficult due to the small size of mice (as discussed in the "Sampling" section).

#### 1. Urea Nitrogen

Urea is produced in the liver as a waste product of protein catabolism (specifically a breakdown product of ammonia). Urea is a small molecule that freely diffuses across cell membranes, and therefore the urea concentration is the same in blood, serum, and plasma.

Traditionally, urea concentrations is measured in terms of urea nitrogen (the amount of nitrogen contained within urea). Determination of urea nitrogen is usually made from serum, but

whole blood can be used (hence the term blood urea nitrogen [BUN]). Quantification of urea nitrogen is based on spectrophotometry assays, measuring the amount of urea that is hydrolyzed by urease.

Frith *et al.* (1980) investigated urea nitrogen levels in BALB/c and C57BL/6 mice. Urea nitrogen levels decreased after 3 months of age but increased again after 12 months in both sexes. In BALB/c mice, levels were higher in males than females, whereas in C57BL/6 mice, females had significantly higher levels than males.

Elevated urea nitrogen (azotemia) can be caused by prerenal, renal, and postrenal conditions. Prerenal causes include increased protein catabolism (such as with inflammation, starvation, and high-protein diets). Renal causes usually are associated with conditions that compromise more than 70–75% of functional renal mass and include conditions such as renal amyloidosis, glomerular immune complex disease, and polycystic disease. Postrenal include any cause that results in obstruction of the lower urinary system. Decreased urea nitrogen is found with disease associated with hepatic insufficiency and low-protein diets.

#### 2. Creatinine

Creatinine is a degradation product of creatine and creatine phosphate and represents an end-product of muscle metabolism. Quantification of creatinine is based on spectrophotometry assays. Baseline serum levels are directly related to muscular conditioning and total muscular mass, which varies between individuals. Pathologically elevated serum creatinine levels are caused by the same prerenal, renal, and postrenal causes that elevate urea nitrogen in serum. Therefore, quantification of serum creatinine offers no interpretative advantage over urea nitrogen (Everett and Harrison 1983).

#### 3. Urinalysis

Proteinuria is a common finding in normal mice, and includes rodent-specific proteins such as uromucoid, small quantities of α- and β-globulins, and a family of prealbumins known as major urinary protein (MUP). The MUP has three electrophoretic variants, designated as MUP-1, MUP-2, and MUP-3, that are under both genetic and hormonal control. A regulatory locus designated *Mup-1* with codominantly expressed alleles *a* and *b* (located on chromosome 4) controls the urinary levels of the three variants. The MUP is synthesized in the liver, secreted into blood, and excreted into urine. Males are have higher levels of proteinuria than are females, with levels of 5 mg/ml, and age-related increases are seen in mice of both sexes. Increases in other urinary proteins have been associated with a variety of renal diseases in mice.

The concentration of urine (amount of solutes dissolved in urine) can be measured by urine specific gravity (USG) or osmolality. Urine concentration in healthy mice is very high. Watts (1975) determined the USG of healthy CBA mice, which ranged from 1.060 to 1.080. The author also found the USG increased from 20 to 80 days of age. The urine concentrating ability of transgenic mice expressing human sickle cell hemoglobin (KOs for mouse Hb) was assessed by Ryan *et al.* (1997). They found that sickled cells caused vascular, tubular, and glomerular changes, as well as corresponding hyposthenuria (4-h water deprived osmolality of affected mice was 807 ± 285 mOsm compared to 1541 ± 360 mOsm in controls).

### I. Electrolytes

#### 1. Sodium, Potassium, Chloride, and Phosphorus

Sodium and potassium levels are easily measured in murine serum using flame photometry (lithium reference) or ion-specific electrodes. Serum sodium levels are slightly higher in mice than in most other mammalian species, with reported values of 174 ± 23 (SD) mEq/l (Finch and Foster 1973), 147 ± 15 (SD) mEq/L (Everett and Harrison 1983), and 155–161 mEq/l (Loeb *et al.* 1996). No differences in serum sodium were seen during aging, between sexes, or among strains. Serum chloride has been measured using mercuric thiocyanate and the chloridimetric or ion-specific electrode techniques, and inorganic phosphorus in mice has been measured using the phosphomolybdate technique. Serum inorganic phosphorus levels decreased as mice aged between 1 and 12 months. This change was documented in BALB/c and C57BL/6 strains, as well as in outbred mice (Loeb *et al.* 1996).

#### 2. Calcium

Total serum calcium reflects both ionized (active calcium) and protein-bound calcium (mainly bound to albumin). Ionized calcium is biologically active in bone formation, neuromuscular activity, cellular biochemical processes, and blood coagulation. Decreased serum albumin levels are also expected to decrease total calcium levels, by decreasing the amount of protein-bound calcium. However, hypoalbuminemia does not result in clinical signs of hypocalcemia.

In mice, total serum calcium has been measured using the sodium alizarin sulfonate technique or atomic absorption spectrometry. Two reports, using different techniques, list similar reference ranges of 9 ± 1 (SD) mg/dl, whereas a third report lists reference ranges of 5.6 ± 0.4 (SD) mg/dl for male and 7.4 ± 0.50 (SD) mg/dl for female albino mice. The latter values reported for male mice were significantly lower than those for six inbred strains of mice in two different age-groups using the same techniques (Quimby 1999b). Likewise, no differences associated with sex were reported by Everett and Harrison (1983), who also examined random bred albino mice. However, Bonella *et al.* (1968) demonstrated significantly higher levels in female Swiss albino mice. Serum calcium levels appear

to decline between 3 and 12 months of age in the BALB/c and C57BL/6 strains as well as in outbred mice. Frith *et al.* (1980) found that female C57BL/6 mice had lower calcium levels than males. Bonella *et al.* (1968) demonstrated significantly elevated calcium levels when blood was collected by orbital puncture versus cardiac puncture.

Hypercalcemia in mice can results as a response to increased levels of PTH (such as with hyperparathyroidism) or PTHrP (such as with certain neoplasms like multiple myeloma). In mice, the response of parathyroid chief cells to hypercalcemia appears different from other species. Grone *et al.* (1992) studied this response in nude mice with PTHrP secreting tumor cells or infusions of PTHrP, and found prominent membranous whorls and increased cytoplasmic area of chief cells in mice with hypercalcemia (17.0 ± 3.1 mg/dl), which marked these cells as distinctly different from parathyroid chief cells of other species. Hypocalcemia can be due to hypoalbuminemia (as previously discussed), renal disease, pancreatitis, and hypomagnesemia. Alcock and Shils (1974) found that mice fed magnesium-deficient diets developed hypocalcemia, as did mice receiving intramuscular injections of heparin.

### J. Other Proteins

Total serum protein has been evaluated in mice using either the Lowry or Biuret methods, although the Lowry method is preferred for analysis of small volume samples. Hypoproteinemia is seen in hepatic injury (also see the "Liver Function Tests" section) or during protein-losing enteropathies or nephropathies. Hyperproteinemia is seen during dehydration. Age-related changes have been described in a number of strains, such as increased total serum protein in older BALB/c and B6 mice and lower levels in DBA/2 mice (Loeb *et al.* 1996).

Each of the major classes of serum protein ($\alpha_1$-, $\alpha_2$-, $\beta$-, and $\gamma$ -globulins) in mice can be distinguished by electrophoresis. Changes in serum levels have been associated with a wide variety of diseases in mice. For instance, hypergammaglobulinemia was found in SCID mice injected with human CAG multiple myeloma cells (Wu *et al.* 2005).

The acute phase reactants ceruloplasmin and fibrinogen have been discussed in the "Serum Proteins" section and CRP and SAP in the "Complement" section. SAA is an acute phase reactant synthesized by the liver and may be induced by the inflammatory cytokines IL-1 or IL-6 (see the "Cytokines and Chemokines" section). Normally SAA is quickly cleaved to a small molecular weight product, but during chronic infection or failure in the degradation pathway, tissue deposition of amyloid may occur. Differences in serum concentrations have been described in various mouse strains and during disease. Commercial RIA and ELISA kits are available for its quantitation in mice (Quimby 1999b).

Alpha-1-fetoprotein (AFP) is encoded by the *Afp* gene which is nearly identical in its organization to the mouse albumin gene, *Alb1*. It is likely *Afp* arose due to gene duplication. AFP is present in fetal serum but the synthesis declines shortly after birth. Serum levels are regulated by two loci, *Afr-1* and *Afr-2*, which are not linked to AFP. The action of these genes may be modified by TGF-β and p53 (Wilkinson *et al.* 2005). Levels in mouse serum have been quantified by immunoelectrophoresis (Zizkovsky 1975) and both polyclonal antibodies and recombinant DNA–derived mouse AFP are available for assay development (Boismenu *et al.* 1997) and are elevated in mice with certain neoplasms and during hepatic regeneration (Jin *et al.* 2005; Quimby 1999b).

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## APPENDIX 6-1
### Abbreviations Used in Chapter

| | |
|---|---|
| 2D-GE | two-dimensional gel electrophoresis |
| 5-HPETE | 5-hydroxyperoxyeicosatetraenoic acid |
| aa | amino acid |
| ABCA1 | adenosine triphosphate-binding cassette transporter A1 |
| ACAT | acyl CoA:cholesterol acyltransferase |
| ACTH | adrenocorticotropic hormone |
| ADP | adenosine diphosphate |
| AFP | alpha-1-fetoprotein |
| AGIF | adipogenesis inhibitory factor |
| AgRP | Agouti-related peptide |
| ALT | alanine aminotransferase |
| AP | alkaline phosphatase |
| ApoJ | apoprotein J |
| AST | aspartate aminotransferase |
| ATP | adenosine triphosphate |
| AVP | arginine vasopressin |
| BCA-1 | B-cell attracting chemokine-1 |
| BMP | bone morphogenic proteins |
| BUN | blood urea nitrogen |
| cANCA | neutrophil cytoplasmic antibody |
| CBG | cortisol-binding globulin |
| CEDIA | cloned enzyme donor immunoassay |
| CETP | cholesteryl ester transfer protein |
| CIC | circulating immune complexes |
| CK | creatine kinase |
| CK-Mt | mitochondrial creatine kinase |
| CNTF | ciliary neurotrophic factor |
| COX | cyclooxygenase |
| CR | complement receptor |
| CRP | C-reactive protein |
| Crry | complement receptor 1 related gene/protein Y |
| CT-1 | cardiotrophin |
| CTLA-8 | cytotoxic T-lymphocyte associated antigen-8 |
| DAF | decay acceleration factor |
| DHT | dihydrotestosterone |
| DME | drug metabolizing enzyme |
| dsDNA | double stranded deoxyribonucleic acid |
| dsRNA | double stranded ribonucleic acid |
| E2 | estradiol |
| ELISA | enzyme-linked immunosorbent assay |
| EMIT | enzyme-multiplier immunoassay technique |
| FasL | Fas ligand |
| FcR | Fc receptor |
| FIA | fluoroimmunoassay |
| FP | prostaglandin $F_2\alpha$ receptor |
| FSH | follicle-stimulating hormone |
| G-CSF | granulocyte colony-stimulating factor |
| GDF | growth differentiation factor |
| GDH | glutamate dehydrogenase |
| GDNF | glial-derived neutrophic factors |
| GH | growth hormone |
| Gld | generalized lymphoproliferative disease |
| GLUT | glucose transporter |
| GM-CSF | granulocyte-monocyte colony-stimulating factor |
| GnRH | gonadotrophin-releasing hormone |
| GOT | glutamic oxaloacetate transaminase |
| GPT | glutamic pyruvic transaminase |
| GTT | glucose tolerance tests |
| H chains | heavy chains |
| HDL | high-density lipoprotein |
| HETE | hydroperoxyeicosatetraenoic acid |
| IAP | intestinal alkaline phosphatase |
| IAPP | islet amyloid polypeptide |
| ICE | interleukin-1 β converting enzyme |
| IDH | iditol dehydrogenase |
| IDL | intermediate density lipoprotein |
| IFN | interferon |
| Ig | immunoglobulin |
| IL | interleukin |
| IP-10 | interferon-inducible protein-10 |
| IRS-2 | insulin receptor substrate-2 |
| I-TAC | interferon-inducible T-cell α chemattractant |
| KO | knockout |
| L chains | light chains |
| LAK | lymphocyte activated killer |
| LCAT | lecithin:cholesterol acyltransferase |
| LDH | lactate dehydrogenase |
| LDL | low-density lipoprotein |
| LDLR | low-density lipoprotein receptor |
| LDV | lactate dehydrogenase-elevating virus |
| LH | luteinizing hormone |
| LIF | leukemia inhibitory factor |
| LO | lipoxygenase |
| LPL | lipoprotein lipase |
| LT | leukotriene |
| LT-α | lymphotoxin-α |
| MAC | membrane attack complex |
| MALDI | matrix-assisted laser desorption/ionization |
| MASP | mannose-binding lectin-associated serine protease |
| MBL | mannose-binding lectin |
| MCP | membrane cofactor protein |
| MCP-1 | macrophage chemattractant protein-1 |
| M-CSF | monocyte colony-stimulating factor |
| MHC | major histocompatibility complex |
| MIS | Müllerian inhibiting substance |

*Continued*

ABBREVIATIONS USED IN CHAPTER—CONT'D

| | |
|---|---|
| MMP | matrix metalloprotease |
| MPO | myeloperoxidase |
| MS | mass spectroscopy |
| MUP | major urinary protein |
| NF-κβ | nuclear factor κβ |
| NIDDK | National Institute of Diabetes and Digestive and Kidney Diseases |
| NK cells | natural killer cells |
| NKSF | natural killer cell stimulatory factor |
| NPY | neuropeptide Y |
| OPG | osteoprotegerin |
| OPGL | osteoprotegerin ligand |
| OSM | oncostatin M |
| OTC | ornithine transcarbamylase |
| OTR | oxytocin receptor |
| PAI-1 | plasminogen activator inhibitor-1 |
| PCNA | proliferating cell nuclear antigen |
| PDGF | platelet-derived growth factor |
| PGs | prostaglandins |
| PIKs | phosphoinositide 3-kinases |
| PLTP | phospholipid transfer protein |
| POMC | precursor proopiomelanocortin |
| PRL | prolactin |
| PRLR | prolactin receptor |
| PTH | parathyroid hormone |
| PTH1R | parathyroid hormone receptor |
| PTHrP | parathyroid hormone–related protein |
| PTX3 | pentraxin 3 |
| QTL | quantitative trait loci |
| RCA | regulator of complement activation |
| RCT | reverse cholesterol transport |
| RF | rheumatoid factor |
| RIA | radioisotopic assay |
| RNP | ribonuclearprotein |
| SAA | serum amyloid A |
| SAP | serum amyloid protein |
| SD | standard deviation |
| SDF-1 | stromal cell–derived factor-1 |
| SDH | sorbitol dehydrogenase |
| SELDI | surface-enhanced laser desorption/ionization |
| SLC | secondary lymphoid tissue chemokine |
| SLD-1 | stearoyl-CoA desaturase-1 |
| SOCS-3 | suppressor of cytokine signaling-3 |
| SRC-1 | steroid receptor coactivator-1 |
| SS | Sjögren's syndrome |
| ssDNA | single-strand deoxyribonucleic acid |
| T3 | triiodothyronine |
| T4 | thyroxine |
| TACE | tumor necrosis factor-α converting enzyme |
| TAJ | toxicity and JNK inducer |
| Tc | cytotoxic T-cells |
| TG | triglyceride |
| TGF-β | transforming growth factor-β |
| TGF-βSF | transforming growth factor-β superfamily |
| TH | T-helper |
| TLR | Toll-like receptors |
| TNAP | tissue nonspecific alkaline phosphatase |
| TNF | tumor necrosis factor |
| TNFRSF | tumor necrosis factor receptor superfamily |
| TNFSF | tumor necrosis factor superfamily |
| TOF | time-of-flight |
| TPC | total plasma cholesterol |
| TRANCE | tumor necrosis factor–related activation-induced cytokines |
| TRH | thyrotropin-releasing hormone |
| TSH | thyroid-stimulating hormone |
| TX | thromboxane |
| UPR | unfolded protein response |
| USG | urine specific gravity |
| VLDL | very low-density lipoprotein |