

# Welfare Issues of Genetically Modified Animals

*Melvin B. Dennis, Jr.*

## Abstract

Genetically engineered animals have opened new frontiers in the study of physiology and disease processes. Mutant animals offer more accurate disease models and increased precision for pathogenesis and treatment studies. Their use offers hope for improved therapy to patients with conditions that currently have poor or ineffective treatments. These advantages have fostered an increase in studies using mice in recent years, a development viewed with alarm by those who oppose the use of animals in research. Scientists point out that the mice are replacing more sentient species, such as nonhuman primates, and are increasing the quality of research being conducted. They assert that study of genetically engineered animals will eventually permit decreases in numbers of animals used in research. Nevertheless, the increase in use of genetically altered animals presents many challenges in reviewing protocols and providing care. Identification and resolution of any welfare problems is a responsibility that is shared by institutional animal care and use committee, veterinary, animal care, and research staffs. To identify potential welfare concerns, a database such as TBASE (<<http://tbase.jax.org>>) can be searched to learn what has been reported for established mutant lines. In addition, newly created lines should be monitored by a surveillance system and have phenotype assessment to identify the effects of altering the genome. Methods of ensuring welfare can include treatment of conditions produced, restriction of gene expression to tissues of interest or to certain time periods, and establishment of endpoints for removing animals from a study before problems appear.

**Key Words:** animal welfare; genetic engineering; homologous recombination; mutagenesis; mutation; transgenic

## Introduction

Modification of the genome of animals has occurred throughout the ages. Initially, changes in genetic composition occurred spontaneously. With the beginning of agriculture, humans exerted influence on the pro-

cess by selecting animals with desirable genetic traits. An animal with a spontaneous genetic mutation that increased feed conversion, boosted milk production, or produced more desirable carcass characteristics would be selected as breeding stock to improve the herd by perpetuating advantageous phenotype changes. More recently, scientists accelerated the mutation process with irradiation and chemical mutagens. Many genetic loci were identified, mapped, and studied using such tools. Genetic predisposition and resistance to diseases were detected.

The era of transgenic animals is a relatively new development that resulted from the injection of foreign DNA into the pronucleus of embryos (Gordon et al. 1980; Palmiter et al. 1982). Offspring expressed the injected DNA construct and passed it on to succeeding generations. The ability to manipulate and study the genome has increased greatly with the subsequent development of gene targeting technology, which allows an investigator to knock out a gene sequence of interest in embryonic stem (ES<sup>1</sup>) cells (Capecchi 1989). Point mutagenesis accomplished by administration of N-ethyl-N-nitrosourea (ENU<sup>1</sup>) is another tool in widespread use to create new disease models.

This article discusses some of the areas of research that are aided by the creation of these animals, welfare issues that have been associated with their production, sources of information to help predict problems, tools available to prevent or limit any welfare difficulties that occur, issues in institutional animal care and use committee (IACUC<sup>1</sup>) review of studies of genetically altered animals, and strategies for addressing welfare problems. Discussion of animal welfare issues generally includes the 3Rs (refinement, reduction, and replacement) of Russell and Burch (1959). Proponents of the use of genetically engineered animals assert that they offer some unique advantages over normal or wild-type animals in achieving the 3Rs. They offer more accurate models that provide more precise data than previously used paradigms, permit the replacement of higher species with less sentient species, and may ultimately reduce the number of animals required to address a particular disease problem. These potential advantages are examined throughout this discussion.

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<sup>1</sup>Abbreviations used in this article: ALS, amyotrophic lateral sclerosis; ENU, N-ethyl-N-nitrosourea; ES, embryonic stem; IACUC, institutional animal care and use committee; p53, short term for Trp53; Trp53, transforming-related protein 53.

## Types of Research

The mapping of the human genome is stimulating one of the most exciting periods in the history of biomedical research. Studies are in progress that could develop new ways to treat disease conditions by altering the genome. The prospect of such treatments has aroused controversy regarding the ethics of potential treatments that would alter the human germ line and, thus, be passed to succeeding generations (Editorial 1999; Willgoos 2001). Until the controversies are resolved, gene therapy trials in humans will probably be limited to modalities that modify genes only in somatic cells. These alterations would not affect offspring of treated patients.

In the near future, germline-modifying therapies will probably be limited to laboratory animals. The ability to manipulate the genome of animals makes them an indispensable component of research involving alteration and modification of genes. It is expected that the research will produce unprecedented breakthroughs in medicine, but a few of the genetically altered animals created may present challenges to the IACUC, the veterinary staff, and others striving to ensure animal well being. Among the areas studied are gene discovery, disease models, test systems, gene therapy, xenotransplantation, and life span extension.

### Gene Discovery

Gene discovery is an area of investigation designed to determine the structure and function of various genes. A closely related area is proteomics, in which proteins produced by genes are studied to discover their action and the interactions with other proteins in the body. Some of these studies are genome driven and use lines of mice created with human or animal genes of interest being either expressed or inactivated and determining the resultant phenotype. Other studies are phenotype driven and use mutagens such as ENU and chlorambucil to produce animals with altered phenotypes. These animals are then studied to determine the gene alterations that produced the particular phenotype.

Because many of the genes to be studied are necessary for fetal development or the basic functions of life, some of the animals produced will likely have health problems and increased lethality. Ensuring animal welfare in some of these newly created lines may require vigilant surveillance and innovative management.

### Disease Models

Application of genetic engineering techniques has enabled the creation of new models for human diseases that have previously lacked accurate spontaneous or experimentally induced animal models. Conditions such as Alzheimer's disease, amyotrophic lateral sclerosis (ALS<sup>1</sup>), and Parkin-

son's disease are subjects of intense efforts to develop new models. One example is a line of transgenic mice, B6SJL-Tg N (SOD1-G93A) 1Gur, which overexpress mutant Cu, Zn superoxide dismutase and are proposed as a model of ALS (Gurney et al. 1994). The mice develop a lower motor neuron syndrome characterized by hind limb weakness at 3 to 4 mo of age that progresses to paralysis by 5 mo. The syndrome is similar to that seen in human patients with ALS. Even research for diseases that previously had many models, such as diabetes mellitus, has been aided by new models developed using transgenic and knockout technology (Wong et al. 1999). The development of improved models that offer advantages over existing ones will continue and will improve the quality of research. Because the lines created will manifest signs similar to those seen in humans affected by the same syndrome, they may present challenging welfare problems.

Work is in progress to develop new models by altering the susceptibility of mice to pathogens of humans. Because the only models for conditions such as viral hepatitis and AIDS have been in nonhuman primates, the use of genetically altered mice offers the potential animal welfare improvement of performing the research in a less sentient species. There is some concern that genetically altered mice might provide a reservoir for these human pathogens and spread infection to the human population. However, the risk from the mice is not greater than from similarly affected nonhuman primates. It is crucial to prevent escape of altered animals to preclude breeding with wild animal populations.

Research into the genetic influence on tumor promotion and suppression is being performed in models created by overexpression or inactivation of specific genes. A group of models has been created by knocking out the transforming-related protein 53 (Trp53<sup>1</sup> or p53<sup>1</sup>) tumor suppressor gene (Donehower et al. 1995; Harvey et al. 1993; Purdie et al. 1994). These lines experience an increased incidence of tumors compared with their wild-type relatives. The frequency, age of onset, and even tumor type varies with different background strains. Commonly used strains for these models include C57BL/6 and 129/P.

It is important to assess the phenotype of each new line created to discover new disease models. An example of the importance of examining each new line is illustrated by a particular strain, C57BL/6J-TgN(LckIL4)1315Dbl, of transgenic mice for interleukin-4 (Lewis et al. 1993). In contrast to lines previously created with similar constructs, one line (no. 1315) became progressively humpbacked starting at 3 to 6 mo of age. Phenotype assessment revealed that the animals were affected with osteoporosis. This unique line became a valuable model for studying the previously unrecognized role of interleukin-4 in osteoporosis.

### Test System Development

Genetic engineering technology is being used to develop improved test systems for examining safety and toxicity of

chemicals, products, drugs, and devices. New mouse models will enable replacement of more sentient species, such as nonhuman primates, in some of these assessments. For example, transgenic mice expressing the human polio virus receptor have been created, and they show promise as substitutes for nonhuman primates to test the safety of attenuated polio vaccines (Ghendon and Lambert 1996). Carcinogenicity testing increasingly uses genetically modified animals, which offer improved systems for evaluation of compounds. Mice with activated *myc*, *ras*, and *neu* oncogenes provide test systems with increased sensitivity for the detection of carcinogenic chemicals.

As gene therapy modalities are developed, new safety testing strategies will need to be developed to assess the effects of the transgene being delivered as well as any toxic effects of vector systems used for delivery. The US Food and Drug Administration recognizes that animal models created through genetic or pharmacological means can be used in an effort to demonstrate that gene therapy products can correct a genetic defect, slow progression of a disease, or alleviate its signs (Pilaro and Serabian 1999).

## Gene Therapy

Treating disease conditions by altering the genome of somatic cells of affected humans and animals is an exciting area of research that provides hope for conditions that presently lack effective treatment modalities. Examples of changes being investigated include replacement of a defective gene with a normal one, insertion of resistance genes, or altering regulatory sequences to turn genes on or off. Research in this area involves creation of laboratory animals with specific genetic defects and then correcting the defects by therapy with normal genes. In reviewing these protocols, it is important to consider the welfare issues of untreated control groups.

## Xenotransplantation

The demand for transplantable organs is fostering research into the creation of animals whose tissues are compatible for implanting into humans. Larger species, such as pigs and baboons, are preferred for development as donors because of the similarity of their organ size to that of humans. However, mice are being used in preliminary studies to assess feasibility and establish procedures to make the animal tissues compatible. These studies might produce animals with modified immune systems that are susceptible to a variety of organisms, some of which are presently believed to be nonpathogenic for the species. These animals may present some novel diagnostic and therapeutic challenges.

## Life Span Extension

As medical research has progressed, the advances in control of disease have resulted in a steady increase in longevity for

humans, with increased health and vigor for the elderly. Recent results with genetically altered animals are raising hopes that perhaps life span can be increased even further. One development that perpetuates such hope is a line of mice with a proto-oncogene at the SHC locus knocked out. (The gene designation SHC was identified by Pelicci and colleagues [1992].) The p66<sup>shc<sup>-/-</sup></sup> mice have increased resistance to oxidative stress and a 30% increased life span compared with wild-type mice (Migliaccio et al. 1999).

The demonstration of such genetic alterations that result in increases in life span is a powerful incentive for research into the genetic control of aspects of the aging process. This area of investigation will remain active in the near future. In this search for genes that will increase longevity, there will also be the creation of some lines with shorter life span and welfare problems.

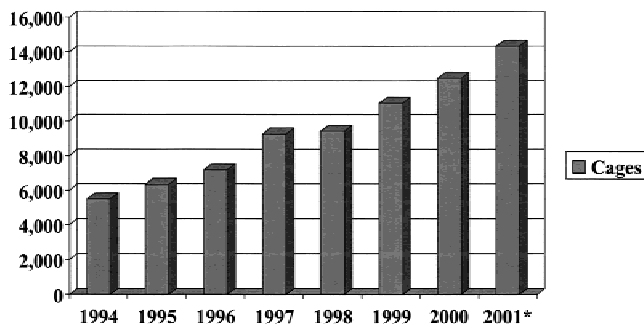
## Identification of Welfare Concerns

### Welfare Issues in Production and Breeding

One of the most controversial and vexing issues associated with the use of genetically engineered animals derives from the sharp increase in numbers of mice used in recent years. Opponents of their use assert that any welfare gains offered by refinement and replacement of other models are offset by the failure to reduce the total number of animals used. The increase in number of mice used in research in recent years can be attributed to intensified efforts in areas that were previously hampered by a lack of adequate models, as well as the development of new technologies.

Although accurate estimates for the number of mice used in research in the United States are not published, I believe that the expansion in mouse populations in centralized facilities at my institution over the past 8 yr is consistent with what is occurring in similar institutions throughout the country. As illustrated in Figure 1, the increase is a total of 161.3% or a rate of more than 23% per year. The increase is due not only to growth in the numbers of animals on studies but also to the large number of animals necessary to create each genetically modified line. In transgenic and knockout lines, these animals include breeding males and donor females needed to produce the embryos for pronuclear injections and for harvesting blastocysts for injection of modified ES cells. Vasectomized males are needed to breed with females to produce pseudopregnant recipients for the altered embryos. In point mutagenesis studies, males are administered ENU to induce mutations in their spermatogonial stem cells. Approximately one in 700 gametes will have a mutation at any given locus, with multiple independent mutations per offspring obtained. Depending on the screening protocol, <1% to up to 10% of animals will have interesting phenotypes (Balling 2001; Nelms and Goodnow 2001). Up to three generations of breeding and screening may be required to detect mutant progeny. A review of the methods required vividly illustrates the large

## MOUSE POPULATION - MEAN DAILY CENSUS



\* 2001 population is mean daily census for two months.

**Figure 1** Average daily census of mouse cages in the centralized animal facilities at the University of Washington from 1993 to 2001. The population increased 161.3% over the period, an average of 23% per year.

number of animals used to establish and characterize the models created by this means (Nelms and Goodnow 2001). Several large ENU screening programs have been established (Justice et al. 1999).

All of the types of animals described above are necessary to produce the genetically modified lines that must then be bred to produce animals to study. They do not, in themselves, produce usable research data. The number of animals not producing data are increased by the small percentage of offspring produced by pronuclear injection, which express the desired genetic alterations in addition to those used in creating crossbred and backcrossed lines of targeted mutations. Even after a line is created, nontransgenic and wild-type littermates may be produced that are not suitable for research or further breeding. Euthanasia is a common fate for these animals.

Another aspect of the numbers issue stems from the fact that once a line is established, it may be necessary to continue breeding animals that have conditions producing welfare problems. Simply to maintain a line, mice with compromised health may be bred but not studied; so these animals also do not produce data directly. A solution for this situation may be the use of embryo or sperm cryopreservation to maintain lines that are not being actively studied (Agca 2000; Critser and Mobraaten 2000; Rall et al. 2000).

The welfare issue of an increasing number of genetically engineered animals may be offset by the pursuit of refinement, another one of the 3Rs of animal use. Genetically altered animals are proving to be more exact or precise models of disease than many of the spontaneous and experimentally induced models used in the past. They may ultimately reduce the number of animals needed for research by producing more accurate data. Already, promising results are being reported in creating genetically engineered

models for diseases in which research has previously been hampered by a lack of reliable models. Examples are discussed below.

When studies involving newly created lines of genetic engineered animals or established lines that are new to an institution are proposed, the IACUC, research team, caretakers, and veterinary staff all are faced with the need to ascertain any special requirements for maintaining the health and well-being of the animals. The process of determining the requirements for newly created lines may be more complicated and difficult than for previously characterized ones. The first questions to be asked should concern what is known about the lines and what are the expected outcomes. The sources of information to answer the questions will vary with the situation.

## Studies of Documented Lines

A series of databases can be searched by an investigator or an IACUC reviewer to ascertain what is known regarding lines of genetically engineered animals that have been characterized previously. The journal *Nucleic Acids Research* annually updates and publishes a list of molecular biology databases (Baxevanis 2001). In 2001, the list included 281 databases, some of which provide information regarding gene expression in a variety of species used in genetic engineering studies including mice, *Xenopus* (frogs), and zebrafish.

One of the most useful databases for investigators and IACUC members is the transgenic animal/targeted mutation database TBASE (<<http://tbase.jax.org>>) (Woychik et al. 1993). It provides information about lines of transgenic and targeted mutant mice. Each entry includes the name of the line and method used to generate it (i.e., pronuclear injection, homologous recombination), DNA construct used, genetic background of host embryo or stem cells, phenotype associated with expression of the genetic change, effects of crossing the line with other mutant lines, how the line is maintained, author's comments, and a contact person to arrange for acquisition of the animals. TBASE also reports the age of onset of changes in phenotype produced by the mutation, progression of disease conditions, and points at which euthanasia should be considered. It can be a source of information about ways to treat animals to prevent or ameliorate progression of signs.

Table 1 contains useful information about selected lines discussed in this article. As an example of how the database is helpful in the identification of welfare concerns, an investigator proposed a study using cystic fibrosis transmembrane regulator gene knockout mice (*CTFR*<sup>-/-</sup>) of the line designated the S489X mutation. A check of the TBASE site revealed that there were four *CTFR* (<sup>-/-</sup>) lines (ID nos. 1094, 1113, 1236, and 3436). One of the lines (ID no. 1113) was designated as the S489X mutation. The line was produced by homologous recombination in E14TG2a ES cells and injection of C57BL/6 blastocysts. The heterozygous

**Table 1 Examples of welfare issues reported in the TBASE database for selected transgenic and knockout lines of mice<sup>a</sup>**

Line name <sup>b</sup>	(+/-) <sup>b</sup>	(-/-)	Lethality	Phenotype (clinical abnormalities)
ApoE (-/-)	A	A	No	Atherosclerosis on high-fat diet, elevated LDL and VLDL, no disease signs on low-fat diet
CFTR (-/-) S489X	WT	A	Postnatal	Runting at birth, most died by 1 mo. Weight loss, abdominal distention, intestinal obstruction and rupture, gallbladder distention and rupture
LDLR (-/-)	A	A	No	Elevated LDL cholesterol levels, no disease signs noted
LIF (-/-)	WT	A	No	Retarded growth, female homozygous -/- are infertile.
RAG-1 (-/-)	WT	A	No	No B or T lymphocytes, no IgM, <sup>b</sup> appear normal to 21 wk, small size
Trp53 (-/-)	A	A	No	Develop multiple tumors (lymphomas and sarcomas) by 6 mo
SOD/G93A	A		Postnatal	Hind limb weakness and paralysis, moribund by 5 mo

<sup>a</sup>The phenotype, whether wild-type (WT) or altered (A), is stated for heterozygous (+/-) and homozygous (-/-) mutants. The degree of alteration of various parameters is described in the phenotype section of TBASE for each line. The alteration is often greater in (-/-) than (+/-).

<sup>b</sup>A, altered; ApoE, apolipoprotein E; CFTR, cystic fibrosis transmembrane regulator; IgM, immunoglobulin M; LDLR, low-density lipoprotein receptor; LIF, leukemia inhibitory factor; RAG, recombination-activating gene; Trp, transforming-related protein; WT, wild-type.

phenotype was described as wild-type, meaning it was unaltered. The homozygous phenotype was reported to be altered, with postnatal lethality. Many deaths occur during the first 5 postnatal days, and very few mice live beyond 30 days. The homozygous mutants are runted and have severe intestinal obstruction leading to death by peritonitis. Because heterozygous animals are phenotypically normal, it is possible to use them for maintaining the line and to produce only the number of affected homozygous animals necessary to accomplish the proposed studies. The reports are not exhaustive and may be dated, so the database should be augmented by information gathered in a current search of the literature. Using a search, the investigator learned that the intestinal obstruction problems in CTFR (-/-) mice can be alleviated by feeding pups a low-residue liquid diet beginning at 10 days of age (Eckman et al. 1995). This example illustrates that TBASE and a search of the literature can be useful initial indicators of the utility of mutant lines and of welfare problems that should be addressed if they are to be used.

Another investigator proposed using mice with the Trp53 tumor suppressor gene knocked out. A check of TBASE revealed numerous lines on several different background strains. It was learned that they all have an increased incidence of tumors, with the type of tumor and age of onset dependent on the background strain of mouse. On a 129/Sv background (now 129/P), the most frequently observed tumor is malignant lymphoma, and testicular tumors are the next most common. The age of the animals at the time of onset of the tumors is 5 wk, and all develop tumors by 6 mo. More than half of the lymphomas involve the thymus and lead to respiratory distress and requiring euthanasia. Similar p53 knockout mice on a background that is 75% C57BL/6 and 25% 129/Sv also develop malignant lymphomas as the predominant tumor. On this background, the age of onset is slightly older, with 74% affected by 6 mo and all affected by

10 mo of age. Testicular tumors are not commonly seen on this background. With this knowledge, it is possible to devise precise monitoring protocols and schedules for each line and to define endpoints.

Other relevant databases available through the Mouse Genome Informatics web site (<<http://www.informatics.jax.org>>) include the Mouse Genome Database (Blake et al. 2001), the Mouse Gene Expression Database (Ringwald et al. 2001), and the Mouse Tumor Biology database (Bult et al. 2001). An investigator or reviewer who uses these resources can access information regarding lines that have been characterized to ascertain problems that have been experienced.

### Studies of New and Uncharacterized Lines

Many of the welfare problems in newly created lines of transgenic animals occur unexpectedly. It is difficult to predict problems because outcomes can vary in different lines produced using the same methodology and DNA constructs due to the randomness of the process of incorporating the DNA construct into the genome. With transgenic mice, the sites of incorporation can vary when one attempts to produce similar lines of animals using identical DNA constructs. A line with one copy of the DNA construct may have a different phenotype from one with two or more copies. In addition, a line with one construct of DNA incorporated into a particular chromosome can have a different phenotype from another line with a copy of an identical construct of DNA incorporated into a different chromosome or even a different location on the same chromosome. Variations in regulatory sequences activated or inactivated, and differences in the background strain of mouse (seen with Tg, ENU, and knockouts) are among other influences that can produce phenotypic dissimilarities in genetically altered mouse lines.

Palmiter and colleagues (1982) illustrated the variability of phenotypes of transgenic mice early in the history of transgenic mouse production by dramatically demonstrating the ability to produce a very large mouse by microinjection of the gene for rat growth hormone into mouse embryos. The size increase that occurred in the transgenic offspring is the phenotypic expression that one would predict as the outcome from overexpression of the growth hormone gene. Unfortunately, subsequent attempts to produce similar lines resulted in lines with a variety of phenotype problems, including liver and kidney failure, increased tumor production, and shortened life span (Wolf and Wanke 1995). These unexpected problems illustrate the difficulty in predicting outcomes when creating new lines of transgenic animals. Examples of similar types of unanticipated outcomes in other lines are described elsewhere in the literature (Dennis 1999, 2000). This randomness of incorporation is not a problem in genetically modified lines produced by other methods. However, there is also a large variation in the phenotypes produced in ENU mutagenesis due to the randomness or variation in which particular genes are mutated.

In newly created genetically altered lines, an effective way to address welfare problems is through a system with two interdependent components. One component is the surveillance for clinical problems by the animal care, research, and veterinary staffs; the other component is the assessment of the phenotype by the research team.

### *Clinical Surveillance*

A surveillance system should include frequent observation of animals to identify concerns early and a reporting system for veterinary evaluation of morbidity and mortality. The program of adequate veterinary care should endeavor to detect and assess illness, physical deficit, injury, or abnormal behavior. In genetic engineering studies, animals should be observed at least daily and perhaps more frequently if uncharacterized lines with problems are being produced. Surveillance is a duty that the animal care, research, and veterinary staffs should share. Newly created lines can present a particularly challenging problem in ensuring that the system is adequate to detect the wide variety of conditions that can occur. Some genetic alterations may produce unexpected syndromes never encountered before. Even seemingly insignificant changes should be studied and documented as potential effects of the genetic alteration. The surveillance program should emphasize vigilance for evidence of anything abnormal.

Although the phenotype changes produced in a particular newly created line may be challenging to predict, as soon as a particular line has been characterized, the types of clinical problems encountered and their time of onset are often more uniform than encountered in their wild-type ancestors. This situation can be helpful in designing monitoring protocols for established lines. A surveillance program should be tailored to ensure that it will identify and address the problems that have been reported in the literature or

databases to affect the line. Often the most important parameters to monitor in a particular line and the crucial time points for increased frequency of observation can be identified. However, there is enough variation that regular examination and vigilance for unforeseen problems is as important as it is for animals in other types of studies. At the University of Washington, the most frequently encountered unanticipated problems are infectious in nature; however, noninfectious problems have also occurred. One example involved apolipoprotein E knockout mice (ApoE  $-/-$ ) on a C57BL/6 background. A database review revealed that these severely hypercholesterolemic mice develop lesions of atherosclerosis but experience no outward signs of disease. However, there is no mention that older animals are consistently found to have thickened, ulcerated skin, with intense pruritis and xanthomas, which are consistent findings in our colony. This case is one of several that illustrate the necessity of monitoring for any conditions that may arise, not only for those already identified.

It is important for a surveillance system to include regular evaluation for murine pathogens and to consider the use of serology, necropsy, and histology. If immune-deficient lines incapable of antibody production are being used, a sentinel program may be necessary to detect the presence of pathogens (Rehg and Toth 1998). In addition, immune-deficient mutant animals may be affected by diseases not commonly seen as clinical problems with immune-competent animals. Inflammatory bowel disease has been reported in both athymic (nude) and severe combined immune-deficient mice (Ward et al. 1996). Rag1 $-/-$  and interleukin (IL)-10 $-/-$  mice have also been shown to be susceptible to experimentally induced infection with severity dependent on the background strain (Burich et al. 2001). Pneumonia due to *Pneumocystis carinii* has been found in mice with the T cell receptor alpha or beta knocked out; whereas, wild-type animals on the same background strain have been resistant. It is worth noting that infectious problems are not commonly noted on TBASE.

### *Phenotype Assessment*

When a new mutant line is created, it is the responsibility of the research team to assess the phenotypic differences between the mutant animals and the wild-type. Of the many protocols proposed, most use observation and minimally invasive tests for general, broad-range characterization of a line. A careful necropsy, including histology when gross lesions are found, should be a component of any basic phenotyping protocol. This is particularly important for animals that are observed to be ill or die unexpectedly. It is possible to follow these methods with more specialized testing to assess particular abnormalities or to assess the potential utility of the mutant line for a particular research area (Becker et al. 1996; Crawley 1999; Rogers et al. 1997; Wood 2000). Behavior phenotyping is an example of more specialized testing, which is being used with increasing frequency. The results of phenotype testing should be available to the

IACUC to review when an investigator requests approval for continued breeding of a line.

## Virus Vectors

Both virus and nonvirus vectors are under investigation as carriers of genetic material for gene therapy for both humans and animals. Intact virus is not used to carry foreign genetic material due to concerns that the vector might produce an infection when injected, or that the transgene might be carried into other animals or humans through horizontal transfer. Concern about infection stems from an incident in which the Moloney murine leukemia virus was used as a gene therapy vector in rhesus monkeys after whole body irradiation. Use of the replication-competent virus was believed to be safe because it is not known to be a pathogen for primates. Three of eight recipients of the gene therapy developed a T cell lymphoma within 7 mo of receiving the virus (Donahue et al. 1992). Although it is not conclusive, it must be suspected that the irradiation altered the monkeys' susceptibility to the virus vector. Due to concerns about such outcomes, virus vectors for gene therapy are normally rendered replication deficient. However, even use of replication-defective viruses is accompanied by concern that they could cause welfare problems in recipient animals. If a helper virus were available, the vector virus might regain the ability to replicate and transmit the transgene, unacceptably, to other animals or humans. Another theoretical problem that must be considered is the effect of an underlying disease on the host's susceptibility to the virus vector.

## Ensuring Welfare

### Containment

One common aim of studies of genetically engineered animals is to learn the consequences of the presence, absence, or alteration of a particular gene. Compromised welfare is usually not intended; however, there are issues that need attention when animals with an altered genome are used in research and testing. Even if the alterations produce no change in the phenotype of the animals, the welfare of feral populations and the environment must be considered. If animals whose genome has been altered by the stable introduction of recombinant DNA into the germ line should escape and breed with feral populations, the environment could be altered and a disastrous situation might be created. To preclude this possible event, altered animals must, therefore, be contained under BL1-N conditions (Federal Register 1994). This degree of containment involves standard microbiological (BL1) practices and limited access to the laboratory when experiments are in progress. Institutions are obligated to take containment measures to prevent the escape of genetically modified animals and establish programs to prevent feral rodents from gaining access to the

animal facilities. Unless breeding or reproductive studies are part of the experiment, a barrier should be provided to separate males and females. As an added containment measure, live mice that are genetically altered should not be released to zoos or pet stores to be used for animal food. To identify animals to be contained, the Guidelines for Research Involving Recombinant DNA (Federal Register 1994) require the permanent marking of genetically engineered animals larger than rodents within 72 hr of birth. If their size does not permit permanent marking, their container should be marked.

The occurrence of immune deficiency is also a potential source of welfare problems that should be addressed by containment. Alterations of histocompatibility and regulatory genes or inactivation of genes required for a particular immune function are among many causes of compromise of immune function in genetically engineered animals. It is also common to breed a particular mutation onto a severe combined immune-deficient (SCID) or athymic (nude) strain of mice. Immune-deficient animals require special living conditions to protect them from organisms that may not be pathogenic to their immune-competent siblings. It is helpful to house immune-compromised animals in ventilated cages with filtered air and to provide sterile cages, bedding, food, and water to prevent occurrence of fatal septicemia. The use of filtered air change stations and protective clothing is also helpful. Even with these precautions, animals may become infected. Cesarean rederivation or continuous antimicrobial therapy may be necessary to control these conditions.

### Treatment

When welfare problems are encountered in lines of genetically engineered animals, treatment is one option that should be considered. An example of successful use of this strategy is the administration of L-DOPA to mice lacking the tyrosine hydroxylase gene in dopaminergic neurones (Zhou and Palmiter 1995; Zhou et al. 1995). Embryonic and neonatal death is the fate of the altered mice unless L-DOPA is administered. Treatment strategies have been used successfully in other disease models for many years. One common example is the administration of insulin to animals with type 1 diabetes mellitus.

### Limitation of Expression

The ability to limit gene expression to certain tissues is a method that has been used to study gene action in both normal and disease situations. A genetic change that would cause serious problems or death when expressed in all tissues can be limited to certain tissues of interest. In this situation, improved welfare is not the primary reason for using the procedure, but it is an incidental benefit. The

absence of expression of the altered genome in the other tissues may protect an animal from phenotype changes that would create welfare problems. One such method of limiting expression is the Cre-*loxP* recombination system (Ray et al. 2000). Cre is an enzyme that catalyzes the removal of a DNA segment that lies between two specific 34 base-pair sequences, termed *loxP*. The system involves creation of two separate lines of mice. In one line, the gene to be knocked out is bracketed with two *loxP* sequences by homologous recombination. The other line has Cre sequences targeted to tissue specific promoters inserted into the genome of the cells of interest. The two lines are then cross-bred to produce a line that has the gene sequence excised in the cells of interest. The gene sequence remains present and functional in the other tissues, although it continues to be bracketed by two *loxP* sequences.

The use of inducible promoters is another method of limiting gene action by turning them on and off for specific time periods. Manifestation of a genetic alteration is needed only for the period of time necessary to accomplish a study. When these techniques are used, genetic manipulations that would potentially result in welfare problems or even death of the animals can now be accomplished and studied. By using a tetracycline promoter (Furth et al. 1994), a gene of interest can be normal while the animal does not receive tetracycline. Gene function can be stopped by feeding the animal tetracycline and restored by discontinuing administration of the tetracycline. Using this system, the animal can have a normal phenotype before and after the study. In addition, Kistner and colleagues (1996) have described a reverse tetracycline system using a doxycycline-inducible promoter. An inserted gene sequence is activated by adding doxycycline to the drinking water. When the doxycycline is stopped, the inserted sequence becomes inactive. Other systems that allow the controlled expression of transgenes include the ecdysone-inducible system (No et al. 1996), the CYP1A1 promoter (Campbell et al. 1996), and a metallothionein promoter (Choo et al. 1986). These systems can even be used to study embryonically lethal conditions (Sarao and Dumont 1998).

## Establishing Endpoints

Humane endpoints for animals used in research and testing are discussed in a recent issue of *ILAR Journal* (Carstens and Moberg 2000; Dennis 2000; Hendriksen and Steen 2000; Morton 2000; Olfert and Godson 2000; Sass 2000; Stokes 2000; Toth 2000; Wallace 2000). In the design phase of a study, consideration should be given to the point at which an animal should be removed from the study. Many genetically manipulated lines lend themselves to the use of objective endpoints because of relative consistency in the occurrence of the conditions that appear, the time point at which they appear, and their severity. Scoring systems have been used with success in studies involving lines showing

this consistency. There are many examples of innovative scoring systems, and some have even been automated for hand-held computers (Hampshire 2001). Some systems, such as body condition scoring, use consistent criteria that can apply to many different situations (Ullman-Cullere and Folz 1999). Other systems may require adjustments to fit individual situations. One example of such alteration involves monitoring of the extent of arthritis in transgenic mice using a score sheet with increasing point values assigned as signs of pain increased in severity. Several animals were observed to show signs of pain before the time when their point totals indicated they should be removed from the study. The problem was resolved by observing that when each individual point value on the sheet was squared, the total score provided a predictable and timely indication of when euthanasia should be considered (Cheunsuk et al. 1999). With any scoring system, there must also be vigilance for unforeseen problems that may not fit the parameters designated for evaluation.

## IACUC Oversight of Welfare Issues

The IACUC is responsible for reviewing and approving proposed animal studies. As investigators create and acquire new genetically altered animals, IACUC members are faced with reviewing proposals that can involve lines with a variety of problems such as premature lethality, altered bodily functions, increased tumor production, decreased disease resistance, altered susceptibility to microorganisms, and many others. In the *Guide for the Care and Use of Laboratory Animals*, one of the topics suggested for review of animal care and use protocols is "Criteria and process for timely intervention, removal of animals from a study, or euthanasia if painful or stressful outcomes are anticipated" (NRC 1996, p. 10). It is customary for the IACUC to ask investigators to list anticipated effects of the proposed manipulations and to describe the planned monitoring protocol for identifying problems.

As discussed above, when new lines are being developed, it may be difficult to predict outcomes accurately. However, there is value in anticipating the possible problems even though the list may require revision as the study develops. A vigilant surveillance system can be helpful in identifying unpredicted events. The endpoints to be used for euthanasia of affected animals should also be listed. It is often possible to select endpoints that are relatively early in a disease process because death is seldom necessary to document the effect or change of a particular genetic change. When problems are encountered, the attending veterinarian and the investigative team should work together to identify ways to prevent or alleviate them. As new methods of limiting the timing and extent of expression of genetic changes are developed, investigators will use them to create more precise and sophisticated models. As has been seen, these refinement methods can also prevent or alleviate many welfare problems.

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