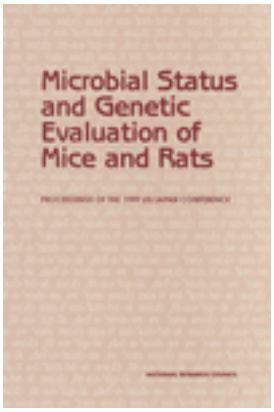

Microbial Status and Genetic Evaluation of Mice and Rats: Proceedings of the 1999 US/Japan Conference

International Committee of the Institute for Laboratory Animal Research, National Research Council

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Microbial Status and Genetic Evaluation of Mice and Rats

PROCEEDINGS OF THE 1999 US/JAPAN CONFERENCE

International Committee of the
Institute for Laboratory Animal Research

National Research Council

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INSTITUTE FOR LABORATORY ANIMAL RESEARCH COUNCIL

Preface

US/Japan meetings on laboratory animal science have been held virtually every year since 1980 under the US/Japan Cooperative Program on Science and Technology. Over the years these meetings have resulted in a number of important documents including the *Manual of Microbiologic Monitoring of Laboratory Animals* published in 1994 and the article *Establishment and Preservation of Reference Inbred Strains of Rats for General Purposes*. In addition to these publications, the meetings have been instrumental in increasing awareness of the need for microbiologic monitoring of laboratory rodents and the need for genetic definition and monitoring of mice and rats.

In cooperation with the Comparative Medicine section of NCRR/NIH, the ILAR Council and staff are pleased to become the host for this important annual meeting and look forward to participating in future meetings. The support and sponsorship of NCRR (P40 RR 11611) in the United States and the Central Institute for Experimental Animals in Japan are gratefully acknowledged. Bringing together the leading scientists in the field of laboratory animal care has resulted in increased understanding of American and Japanese approaches to laboratory animal science and should continue to strengthen efforts to harmonize approaches aimed at resolving common challenges in the use of animal models for biomedical research and testing. This effort to improve understanding and cooperation between Japan and the United States should also be useful in developing similar interaction with other regions of the world including Europe, Australia, and Southeast Asia.

John Vandenbergh, *Chair*
International Committee of the Institute
for Laboratory Animal Research

Contents

Opening Remarks

Judith L. Vaitukaitis
Director, National Center for Research Resources
National Institutes of Health
Bethesda, MD

We are here today because we share enthusiasm for establishing the highest possible global standards for laboratory animal models, which we need to share universally. Sharing is not only a cost-effective approach but is also a way to acknowledge that biomedical research is now on a global scale. This global venue for biomedical research enhances the probability that scientific advances to improve human health will reach the people of all nations. As we meet, valuable laboratory animal models are being exchanged among biomedical investigators at research institutions worldwide.

Our collective attention to carefully characterizing and continuously monitoring the quality of our research animals contributes to establishing worldwide standards that will benefit all users. As the 20th century draws to a close, we salute the vision of Drs. Held, Allen, Nomura, Kaguiama, and others who, on behalf of their respective countries, had the foresight to focus on quality control standards two decades ago. The common denominator for the ongoing collaborations has been the dedication to improving and sustaining the quality of the laboratory animal scientific infrastructure.

The genome sequencing efforts for several species will provide the basis for well-characterized animal models for study into the next millennium. Laboratory animal models are invaluable for investigators to discern mechanisms of disease and develop novel approaches to prevent, control, or cure diseases with genetic factors that contribute to human disease or its susceptibility. The genomes of several important animal models already have been sequenced, and human genome efforts are rapidly moving forward. These activities further emphasize the need for well-defined laboratory animals that can provide the vital link between

basic research and patient studies. To this end, it is crucial to establish standards for monitoring genotype, phenotype, microbial status, and the environmental quality of the mouse and rat. Genetically altered rodent models are even more susceptible to their microbial environments than their genetically intact litter mates. This simple fact is not widely appreciated, however. At this meeting today, we will exchange information and further strengthen efforts in these critical areas.

Standardized databases are necessary for cataloging the many validated genetically altered rodents to prevent unnecessary duplication of this research effort globally. Complementary data—normal gene and altered gene sequences of induced mutants and their phenotypes—must be captured in databases for seamless access to facilitate research as the demand by researchers for high-quality genetically engineered mice and rats steadily increases. There is a great need for international collaboration in database design, data entry standardization, and management.

We must also train more experts in rodent pathobiology. Pathobiologists are essential for characterizing the impact of genetic alterations on phenotype. To enhance career development in this area, the National Institutes of Health (NIH) is extending support for training and career development opportunities for veterinarians in laboratory animal pathobiology. This effort is expected to significantly increase the quality of laboratory animal model characterization and complement the effort of other scientists working with these models.

In recent years, NIH has initiated activities to position the rat model parallel with that of the mouse. Briefly, the trans-NIH mouse research priorities focus on sequencing of the mouse genome, functional analysis of induced genetic defects, and development of repositories and databases. In addition, several NIH institutes and centers are expanding support for the career development and retraining of experts in rodent pathobiology. NIH priorities include mapping and sequencing the mouse genome, establishing mutagenesis and phenotyping centers as well as expanding mutant mouse regional resource centers, and developing or expanding databases to facilitate access to appropriate animal models and provide relevant information about those genetic models.

The rat model is also considered a primary biologic discovery tool and a principal model system for assigning functions to genes. It particularly offers an excellent model system for toxicology and pharmacology studies. Rat model advocates believe the interaction of rat physiologists with mouse geneticists will enhance the possibilities of discovering and characterizing new genetic models of human disease. Again, the pathogen status and quality of these research animals are paramount to research integrity.

In the next century, we and others may look back on today's meeting and earlier efforts and wonder how the participants of this US-Japan effort had the foresight to address key quality control issues. The issues may have seemed mundane to some but will be viewed as absolutely essential to the significant

progress in defining the key role that laboratory animal models contribute to understanding the mechanisms of human disease and developing ways to improve human health—not only in the United States and Japan, but globally as well.

I again commend your efforts to further scientific progress through this exchange program, established many years ago by the governments of the United States and Japan. We must continue to safeguard our valuable and fragile laboratory animals by microbiologic monitoring for major infectious agents, improving diagnostic techniques for disease, training more pathobiologists, and developing more relevant databases and tools to mine data and increase access to information and other essential research resources. May our exchange today yield the knowledge needed to fulfill the world health promise of tomorrow.

Opening Remarks

Shin-Ichi Ota
Director
Division of Science Information
Ministry of Education, Science, Sports and Culture
Tokyo, Japan

It is unnecessary to repeat the many positive consequences of this cooperative program since its beginning in 1980. However, I would like to mention the following noteworthy publications that have resulted from our meetings:

- *Manual of Microbiologic Monitoring of Laboratory Animals*, 1st and 2nd eds. (USPHS/NIH 1986, 1994).
- *Establishment and Preservation of Reference Inbred Strains of Rats for General Purpose Use* (Nomura and Potkay 1991).

International standardization of rats was undertaken first by this US/Japan Cooperative Program and subsequently by the International Council for Laboratory Animal Science (ICLAS). These activities have reached a global scale with the ICLAS International Rat Genetic Nomenclature Committee, which met first in 1994 in Sapporo, Japan; second in 1996 in Toulouse, France; and third in 1998 in Halifax, Canada.

Recently, biotechnology has made remarkable progress using transgenic animals, and international collaborative studies have used these animals widely. International harmonization of drug safety data for new drug development is also under way. Therefore, international standardization of the quality of laboratory animals as tools for obtaining reproducible data has become even more important.

The composition and location of US/Japan meetings have recently changed slightly. Earlier meetings were hosted by the National Center for Research Resources of the National Institutes of Health. For the last 2 years, we have met

at the National Academy of Sciences, where the Institute for Laboratory Animal Research has been our host. I sincerely hope that we will continue having these meetings and will expand our discussion topics in the future. Since 1980, this meeting has been held only in the United States. For the US/Japan Science and Technology Cooperative Program to pursue the mutual benefit for which it was established, I believe that some future meetings should be held in Japan or, if Japan is not possible, in Hawaii.

Finally, I sincerely thank Dr. Judith Vaitukaitis and Dr. Ralph Dell as well as all US participants for their efforts in organizing this meeting.

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Introductory Comments on Microbiologic Testing of Laboratory Mice and Rats: Uniformity of Results

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Retired, National Institutes of Health and
Microbiological Associates, Inc., Bethesda, MD

Although the search for ways to improve the uniformity and correctness of test results produced by animal testing laboratories worldwide would appear to be a relatively simple aim, it involves difficult issues because laboratories around the world are managed differently. For example, such laboratories use a variety of tests for a given agent, a multiplicity of testing reagents of varying quality, and many types of equipment; and they have personnel with very different levels of training and expertise. These variables are not likely to be eliminated in the near future because of differences in resources among countries, cultural nuances, resistance to change, and so forth. However, we can still strive for more uniformity of testing even if the efforts must begin focally and spread to other areas at a later time.

Initiatives of this type are no doubt occurring in a number of countries. In the United States, a few efforts have been made but have not been carried very far. The more formal approaches that come to mind include the program that Dr. Dennis Stark began at Rockefeller University, where multiple laboratories are invited to test a single sample and then compare results. Another approach was the development of 25 "monospecific" reference antibody reagents for use in helping to standardize antibody tests for infectious agents of mice and rats. Production of the reagents was accomplished by the combined efforts of the American Committee on Laboratory Animal Diseases (ACLAD) and Microbiological Associates (now BioReliance Corp).

Our speakers are eminently qualified to discuss this subject and give their perspectives.

Development of a Performance Assessment Program for Research Animal Diagnostic Laboratories and Defining Microbiologic Testing Standards

Lela K. Riley
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University of Missouri,
Columbia, MO

Efforts are being made in this country to develop an expanded performance assessment program (PAP) for research animal diagnostic laboratories to help ensure uniformity of diagnostic testing. All of us who are involved in health monitoring of laboratory animals recognize the importance of accurately assessing the health of animals to be used in biomedical research. Clearly, microbial infections can and do interfere with, alter, and even invalidate research studies by altering the physiology of animal models. Thus, it is critical that we as diagnosticians provide accurate health assessments of laboratory animals to the veterinarians who care for these animals and investigators who use these animals.

We also recognize that advances in genetic manipulation of rodents have led to an explosion in the numbers of transgenic and knock-out mutant mouse and rat models. Because these mutant mice and rats represent extremely valuable models to researchers worldwide, these rodents are frequently shared among researchers in institutions in United States, Japan, and other countries. With this increased trafficking of rodents, the importance of accurate health monitoring has never been greater.

As Dr. Shek has described, multiple assays are available for diagnostic evaluation of animals, each with their own advantages and disadvantages. In addition, individual diagnostic laboratories have developed their own reagents and methodologies for performance of diagnostic tests. Although different methods may be used by different laboratories, the important issue is not what test is used or what reagents are used but instead, the accuracy of results. To ensure that test results are accurate, several major diagnostic laboratories in the United States have recently initiated efforts to develop a comprehensive performance assess-

ment program for diagnostic testing of laboratory animals. A comprehensive program such as this currently does not exist in this country.

The following institutions and companies are presently involved in initiating this effort: Anmed Biosafe, Inc.; Bioreliance; Charles River Laboratories; Harlan Sprague Dawley, Inc.; The National Cancer Institute; and our laboratory at the University of Missouri. This group is not intended to be exclusive. It simply represents institutions and companies that operate research animal diagnostic laboratories and are committed to high-quality testing of laboratory animals.

This group has met on several occasions to set goals, establish priorities, and begin to organize the framework for a comprehensive performance assessment program. The goal of the program is to develop a comprehensive performance assessment program that will distribute unknown specimens to participating diagnostic laboratories for testing. Testing results will then be collected and analyzed by an unbiased external third party. Finally, the testing laboratories will be apprised of expected results for each specimen distributed.

If testing laboratories correctly identify a microbial contaminant or infection, the laboratory is provided additional validation of its testing reagents and methods. If test results do not match expected results, the laboratory is alerted to the problem and can modify its testing procedures.

Several fundamental principles will be emphasized in this program.

1. The program will be comprehensive and will assess all types of diagnostic test methods including serology, bacteriology, molecular techniques such as polymerase chain reaction-based diagnostics, parasitology, histopathology, and new test modalities as they become available.

2. Specimens distributed to participating laboratories will be well defined and will be made by experimental infection of rodents with well-characterized microorganisms. Alternatively, specimens may be collected from natural outbreak infections and documented extensively to identify the causative agent. Methods for documentation will include testing by a battery of sensitive and specific tests. For example, serum collected from an outbreak of natural infection will be tested by multiple serologic assays, including enzyme-linked immunosorbent assay, immunofluorescent assay, hemagglutination inhibition assay if appropriate, and Western blot analysis. Only if results of all tests are consistent for a specific etiologic agent or disease will the specimen be distributed to laboratories participating in the PAP.

3. Distributed specimens will be relevant to infections found in laboratory animals, thus allowing diagnostic laboratories to focus attention on types of microbial infections that they are likely to encounter in laboratory animals. Infectious agents that are currently the most prevalent in laboratory animals will be emphasized. As new emerging agents are identified and diagnostic tests are developed, we anticipate including specimens that will test the ability of diagnostic laboratories to detect these newly recognized agents and infections.

Currently, this group is developing standard operating procedures for preparation and validation of specimens, storage of specimens, shipment of specimens to participating laboratories, and reporting of results. We are also currently in the process of identifying an external party who will distribute the specimens and collect and analyze reports from participating laboratories.

We plan a two-phase implementation of this PAP. In phase I, we anticipate distribution of specimens to a small group of laboratories including those involved in the planning phases of this program. This phase is designed to allow identification of problems in any aspect of the program including preparation of specimens for testing, shipment of samples and reporting procedures. Procedures will be modified as needed to eliminate any identified problems. The goal is to initiate phase I of the PAP by July of 2000.

Phase II will be initiated in 2001 and will expand the program to allow any laboratory to participate in the program, including diagnostic laboratories in the United States, Japan, and other countries. Fees will be assessed from participating laboratories to recover costs associated with production and shipment of test specimens and collection and analysis of test reports.

As soon as this PAP is operational, results obtained from laboratories participating in the program should yield uniform testing results among diagnostic laboratories and provide users confidence in testing results. I believe this comprehensive PAP will benefit both diagnostic laboratories and the users and producers of laboratory animals. It will benefit diagnostic laboratories by providing an additional validation of their testing methods and reagents, and it will benefit users by ensuring that test results among participating laboratories are as consistent and uniform as possible.

Nevertheless, it is important to remember that diagnostic testing does not always yield unequivocal results. Certain samples may contain interfering substances that confound testing. These interfering substances may be due to the health of the animal at the time the sample was collected, the strain of the animal, prior experimental manipulation of the animal, the procedure used for collection of the sample, or how the sample was stored between the time it was collected and the time it was tested. Therefore, it is unrealistic to think that every sample will yield unequivocal results.

It is important to remember that accurate health monitoring requires the involvement of both the diagnostic laboratory and the laboratory animal veterinarian and his/her staff. Laboratory animal veterinarians and professionals must ensure that the appropriate number of animals are tested to provide a high confidence level of detecting the infection. For microorganisms that have a low incidence of infection, this may mean testing a large number of animals.

Laboratory animal veterinarians must also ensure that specimens are collected from animals of the appropriate age and that samples are collected and stored appropriately before shipment to a diagnostic laboratory for testing. If sentinels are used to evaluate the infection status of an animal colony, it is

essential that the sentinel monitoring program is appropriately established and performed.

Another issue that faces laboratory animal veterinarians and diagnostic laboratories is defining the microorganisms and infections for which we will test. Although it is absolutely critical that one ensures the health of animals within a facility and documents the health status of animals being imported into a facility to prevent outbreaks of disease, it is inappropriate to establish a list of organisms that are unacceptable. Microorganisms that are ubiquitously unacceptable in one facility may cause no problem in another facility. For example, *Corynebacterium bovis*, the agent of hyperkeratosis in nude mice, is carried on the skin of immunocompetent haired mice and causes no adverse effects in these mice. In a facility with only immunocompetent mice, this agent poses no threat. However, in a facility with nude mice, the presence of this organism poses a real and major threat. Thus, the organisms to test for must be determined on the basis of the individual situation.

One possible approach to diagnostic testing of animals to be imported into a facility is for the receiving institution to request a recent health report from the donating facility. If the health report is not recent (within the last 1 to 2 months) or if any agent of concern for the recipient institution has not been evaluated, then the recipient institution should request that the donating institution perform additional health evaluations. Cost of additional health monitoring should be negotiated between the donating and recipient institution depending on the circumstances. Alternatively, the recipient may import and strictly quarantine the animals until it can be determined that their health status meets the standards of the facility.

In summary, the importance of accurate health monitoring of laboratory animals is well recognized. I believe that diagnostic laboratories are committed to providing the best testing possible. To this end, several of the testing diagnostic laboratories in this country are involved in establishing a comprehensive PAP. We anticipate that this program will be available to diagnostic laboratories worldwide by 2001 and that this program will benefit the entire laboratory animal and biomedical research communities by ensuring uniform testing capabilities among participating diagnostic laboratories. We believe that joint efforts between diagnostic laboratories and laboratory animal veterinarians and professionals can achieve the common goal of healthy animals being used in biomedical research.

Standardization of Rodent Health Surveillance: Regulation Versus Competition

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INTRODUCTION

Standardization may result from regulation or "recommendations" (in quotes because the recommendations are often followed as if they were regulations). In Europe, an attempt is being made to standardize rodent health monitoring through recommendations promulgated by the Federation of European Laboratory Animal Science Associations (FELASA) (Rehbinder and others 1996). These recommendations, which include lists of infectious agents and standard report formats by species, were developed to simplify the evaluation of rodent health status, irrespective of source. However, some have misunderstood the purpose of the recommendations by concluding that rodents infected with any of the microorganisms on the FELASA reference list are not suitable for use in research. Such misunderstanding underscores the danger of presenting simple lists of infectious agents to people who do not have a general understanding of laboratory animal microbiology. The relevance of lists of microorganisms, such as those published by FELASA, is further compromised when they are not regularly updated to include the latest findings in laboratory animal microbiology, a very active field of research. For example, helicobacters are not included in the FELASA list. Even recently prepared lists will be more applicable to certain situations than to others. In addition, they inevitably reflect the bias and limited knowledge of the cadre who create them.

Another approach is to standardize to the diagnostic reagents and methods of a reference laboratory. Dependable reagents from a trusted and well-known source can be especially valuable when the relevant resources and technical

expertise are not available, especially if the reagents are provided in a simple-to-use kit format. Standardized reagents and assay methods might also lead to fewer discrepancies in the results from different laboratories, but such strict standardization is not without significant risks. No assay is completely sensitive and specific. Often the deficiencies in an assay become known only when comparative testing is performed in multiple laboratories that use different reagents and test methods. In the 1980s, the first evidence for the existence of additional rodent parvovirus serotypes came from laboratories employing indirect immunofluorescence assays (IFA) to detect rodent parvovirus antibodies, instead of the more commonly used hemagglutination-inhibition tests (HAI) and enzyme-linked immunosorbent assays (ELISA). The better sensitivity of the IFA for detecting cross-reacting parvovirus antibodies is related to the nature of the IFA antigen, which is composed of infected cells that contain large amounts of the highly conserved and, hence, cross-reactive nonstructural (NS) parvoviral proteins. Certainly in this case, had all laboratories been using recommended assays and reagents, the discovery of the new parvovirus serotypes (i.e., mouse parvovirus [MPV] and rat parvovirus [RPV]) would have been delayed substantially.

In the United States, there is minimal governmental regulation of laboratory animal testing, although the USDA does license veterinary diagnostic test kits, including those developed to test rodents. Despite the dearth of regulatory oversight, there have been continual and substantial improvements in the health of laboratory rodents and the breadth and quality of rodent diagnostic services. The contention made here is that these improvements and de facto standardization have come about, and will continue to occur, because of competition among laboratory animal suppliers, diagnostic laboratories, and researchers. Furthermore, regulations and recommendations implemented by governmental agencies and professional organizations might impede the incorporation of recent research advances into current laboratory animal husbandry and health surveillance practices.

ANIMAL HEALTH

Husbandry

As noted, the quality of specific-pathogen-free (SPF) laboratory rodents supplied by major animal breeders has improved steadily and dramatically in the United States with minimal government regulation. For example, most vendors have switched in recent years from raising severe combined immunodeficiency mice and athymic nude mice in barrier rooms to raising them in isolators in which a restricted microflora, free of opportunistic pathogens, can be sustained. This costly change was not made in response to a mandate from a government agency or professional organization. Rather, it was implemented to meet the demands of researchers for healthier animals and to stay competitive.

As investigators have become more cognizant of the expanding body of scientific studies showing the complications to research that are caused by adventitious infections, biosecurity has improved at research facilities as well. Many institutions have adopted the use of microisolation cages, and it appears that the prevalence of adventitious infections has dramatically decreased at these institutions.

Surveillance

Without prescriptions from governmental agencies, commercial and noncommercial laboratory animal vendors are screening their colonies for largely the same infectious agents, including most exogenous viruses, primary and opportunistic bacteria, and ecto- and endoparasites. With regard to sample size and frequency, it appears that serology for viral antibodies is performed on a monthly to quarterly basis, whereas other types of health monitoring, bacteriology, and parasitology are performed on a quarterly basis. Many factors such as husbandry practices and the historical incidence of contamination, however, affect whether sample size and the frequency of testing are adequate. Statistical models that take into consideration the effect of current husbandry practices (such as the use of microisolators and sentinels kept on pooled, soiled bedding) on sampling are just not available. Therefore, governmental and professional organizations have no sound basis from which to mandate or recommend sample size or sampling frequency.

Results Reporting

The reporting of results has also become standard among laboratory rodent vendors. Generally, vendors report health surveillance results by room and species. The panel of microorganisms included in the reports is largely consistent from vendor to vendor. Reports are generally divided into three sections: serology, bacteriology, and parasitology. Most suppliers have two report categories, specific pathogen free (SPF) and additional agents. SPF reports include those agents that must be excluded from a colony because of documented health and research effects. The additional agents report may include opportunists and other agents of interest to researchers. Results are presented as the number positive over number tested. Reports typically show cumulative data, perhaps over a 12-month period; the most recent test results may also be presented. Sometimes animal strains in the room are listed in the report.

Plan of Action

When an adventitious infection is found, a vendor's action is largely determined by customer requirements and the microbial status of competitor colonies.

A vendor might compile certain lists of agents for which immediate or planned depopulation is mandated because of pathogenicity, research effects, or customer preferences. We recently detected seroconversion to Theiler's mouse encephalomyelitis virus (TMEV) in a rat colony. Essentially nothing is known about the cause of TMEV seroconversion in rats. We suspect a related picornavirus is responsible for seroconversion, but our attempts to isolate or detect virus by animal inoculation or polymerase chain reaction (PCR), respectively, have been unsuccessful. Despite this and a dearth of information in the laboratory animal science literature, once informed of our serologic findings, customers chose not to receive rats from the affected colony. Hence, customers essentially determined that we had to eliminate the colony. Of course, a vendor's actions also depend on financial considerations. If exclusion of a particular microorganism becomes important to the research community but all of a supplier's colonies are infected with that agent, the supplier cannot be expected to immediately depopulate all affected colonies. However, we have observed in many instances that competition from suppliers with colonies free from infection with a particular pathogen will cause others to replace their infected colonies.

HEALTH SURVEILLANCE

Methodologies

All or most diagnostic laboratories have by now converted to using for serology the sensitive, nonradioisotopic, solid-phase immunoassays developed in the 1980s, such as the ELISA and IFA. Among the molecular, or DNA, methodologies that have recently been applied to infectious disease diagnosis, the PCR has become the most popular. In rodent diagnostics, the University of Missouri Research Animal Diagnostic and Investigative Laboratory has led the way and has the largest panel of PCR assays for rodent infectious agents. Most other diagnostic laboratories, including ours, are following suit by developing PCR for viruses and other microorganisms.

From the comparative results of rodent diagnostic quality control program, we know that there can be great disparities among the test results reported by different laboratories. Comparative serology results recently reported by the European Laboratory Animal Health Monitoring Club, however, showed substantial agreement among laboratories, albeit for a limited number of viruses and other microorganisms (Dix and Needham 1996).

Microorganisms

Emerging rodent pathogens are continually being discovered by laboratory animal microbiologists. Without regulation or recommendations, how do rodent diagnostic laboratories respond to these findings? In the 1980s, the important

newly discovered agents were MPV and RPV, originally known as the "orphan" parvoviruses (Jacoby and others 1996). The pathogenesis of MPV in particular has been elucidated at Yale's Section of Comparative Medicine, which it is worth noting received substantial support in this effort from a commercial vendor. At this time, most if not all laboratories offer serology for MPV and RPV antibodies, primarily by ELISA with recombinant antigens and by IFA.

More recently, research done at various institutions has shown that infections with certain species of *Helicobacter* may cause disease, especially in immunodeficient mice. Without any regulations or recommendations requiring them to do so, most rodent diagnostic laboratories in the United States (and in Europe and Japan as well) quickly developed and began offering *Helicobacter* PCR assays.

SUMMARY

In the United States (and I would argue in Europe and Japan as well), the main motivation for standardization among the suppliers of rodents and diagnostic services has been competition and not government regulation or the recommendations of professional organizations. There is strong competition among microbiologists, laboratory animal suppliers, and diagnostic laboratories to discover and publish on important etiologic agents, to provide the highest quality SPF rodents, and to offer the most complete and accurate testing services, respectively. Laboratory animal suppliers have had to conform to the de facto standards that are set by their competitors and the demands of the research community. Consequently, there is little variation among suppliers of the excluded infectious agents that define rodents as SPF. To keep up with their competitors, diagnostic laboratories have had to quickly adopt the latest assay methodologies and to add tests for emerging pathogens. Concordance of the results reported by different laboratories for assays to diagnose common infections is good. Nevertheless, a quality assurance program to assess the accuracy of laboratory results, such as the one discussed in this meeting by Dr. Riley, is sorely needed.

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Factors Causing Difficulties in Uniformity of Results Among Testing Facilities in Microbiologic Monitoring of Laboratory Animals

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Central Institute for Experimental Animals
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It is important to select test items for harmonization of microbiologic monitoring of laboratory animals, but selection alone is not sufficient. These tests will not be effective unless the methods, including sampling, testing techniques, and expression of results, are uniform and the items and methods are considered as a set. I believe that many factors are involved in disparate results among different testing facilities. At the Central Institute for Experimental Animals (CIEA), we have encountered discrepancies in results among testing facilities involving *Pasteurella pneumotropica, Clostridium piliforme* (Tyzzer's organism), and Hantavirus.

DISCREPANCIES IN RESULTS

Pasteurella pneumotropica

P. pneumotropica is a pathogen that has been found in the respiratory tract of mice and rats, but until recently, it has not been considered important as a pathogen. In culture tests, discrepancies in results have often been observed. To test for this organism, we coat swabs from the trachea and conjunctiva on horse blood agar plates and ultimately identify suspected colonies by Gram staining, testing of characteristics by the API system, and DNA testing by polymerase chain reaction (PCR). Identification is often difficult, however, because there are many analogous bacteria with slightly different properties from this organism. Even

when kits are used, difficulties in accurate identification often arise in testing facilities with little experience or in animal facilities with small scale testing.

In one facility in a medical school where *P. pneumotropica* was listed in the quarantine protocol for the barrier facility, tests were performed by PCR because of difficulty in identification by the conventional method. A swab from the conjunctiva was cultured in a liquid medium for bacterial growth, and the specific sequence of 16SrDNA of this organism was then detected by PCR. This method was used for quarantine because good results had been obtained in an experimental study and live animals could be tested. However, when the method was used in actual quarantine, the number of positive animals immediately increased and the introduction of new animals became difficult. When we tested some of the animals that were positive in the first test, we obtained consistently negative results. It is evident that results of bacteriologic tests using cultures will show discrepancies if there are differences in sampling sites, test methods, and identification criteria.

We also have encountered prolems in antibody testing, which we use for microbiologic monitoring of laboratory animals. The ICLAS Monitoring Center typically uses enzyme-linked immunosorbent assay (ELISA) as a screening test followed by the indirect immunofluorescent antibody (IFA) test or another method (such as the immunoblot method, hemagglutination inhibition test, or neutralization test) on serum suspected of being positive. When the sample is positive in both tests, it is evaluated as positive.

Clostridium piliforme (Tyzzer's organism)

Tyzzer's disease, which is characterized by diarrhea, focal necrosis in the liver, or death, has recently occurred in rats and rabbits at several breeding facilities in Japan. Sporadic cases of laboratory animals positive for this organism have also appeared in Japan.

Because it is difficult to culture this organism, we commonly detect the infection by using an antibody test such as complement fixation (CF), IFA, or ELISA. Generally, in antibody tests on mice and rats, the CF method is less sensitive and results in many false negatives, whereas ELISA is very sensitive and seldom results in false-positive cases.

At the ICLAS General Assembly held in May 1999, it was reported that a breeder of laboratory animals who first obtained antibody-positive test results from a testing facility and destroyed the animals later learned that the test results were incorrect, which caused a major loss. We also had tested these sera and had obtained negative results, and I have heard that these samples were also found to be negative when tested by an American testing facility.

The ICLAS Monitoring Center distributes an ELISA antibody test kit for Tyzzer's disease. Serum samples suspected of being antibody positive are sent to the Center where a confirmation test using IFA is performed. According to the

confirmation test, approximately one fourth of the specimens have actually been positive. I suspect that major problems have not occurred in Japan concerning serologic checking of this organism because this confirmation test is performed.

With the usual serologic test methods for Tyzzer's disease, ELISA and IFA, there appear to be no marked differences in use among testing facilities. However, a detailed investigation revealed differences in strains, antigen preparation, and evaluation of results. It had been found previously that false positives occurred even in Tyzzer's disease tests by ELISA. False-positive reactions can be divided into nonspecific reactions that occur in systems and specific reactions due to cross-reactions with organisms having common antigens. The following example pertains to the latter type

As shown in Tables 1 and 2, we checked serum samples that were ELISA positive and IFA negative for Tyzzer's organism. All of these rat sera reacted with *Clostridium spiroforme* and most rabbit sera reacted with *Clostridium clostridiforme*, the two clostridial species that are components of these animals' intestinal flora. Results indicated that cross-reactions with *C. spiroforme* in rats and *C. clostridiforme* in rabbits are one of the causes of false-positive reactions

TABLE 1 Cross-Reaction in ELISA[a] for Detection of Antibody to *Clostridium piliforme* (Tyzzer's organism) in Rat Sera[b]

	Tyzzer's Organism		*Clostridium clostridiforme*		*Clostridium spiroforme*	
Sample no.	ELISA	IFA[a]	ELISA	IFA	ELISA	IFA
A - 1	0.523	–	–	–	1.868	+
2	0.435	–	–	–	1.096	+
3	0.704	–	–	–	0.815	+
4	0.619	–	–	–	1.254	+
5	0.577	–	0.332	–	1.743	+
6	0.600	–	1.084	+	0.509	+
B - 1	0.691	–	–	–	1.946	+
2	0.401	–	–	–	1.747	+
3	0.757	–	–	–	1.348	+
C - 1	0.301	–	0.334	+	2.408	+
2	0.309	–	–	–	1.910	+
3	0.348	–	0.626	+	1.751	+
4	0.379	–	0.409	+	1.582	+
D - 1	0.699	–	0.315	–	2.358	+
2	0.820	–	0.656	–	2.554	+
3	1.237	–	–	–	1.517	+

[a]ELISA, enzyme-linked immunosorbent assay; IFA, immunofluorescent antibody.
[b]ELISA: OD492 value of 0.3 or higher was regarded as positive; IFA: Antibody titer 1:10 or higher was regarded as positive.

TABLE 2 Cross-reaction in ELISA[a] for Detection of Antibody to *Clostridium piliforme* (Tyzzer's Organism) in Rabbit Sera[b]

	Tyzzer's Organism		*Clostridium clostridiforme*		*Clostridium spiroforme*	
Sample no.	ELISA	IFA[a]	ELISA	IFA	ELISA	IFA
E - 1	1.934	–	–	–	2.523	+
F - 1	0.320	–	0.499	+	–	–
G - 1	1.109	–	1.161	+	1.069	–
H - 1	0.449	–	0.378	+	0.599	+
I - 1	0.409	–	1.151	+	3.000	+
J - 1	0.495	–	1.585	+	–	–
K - 1	0.812	–	1.722	+	–	–

[a]ELISA, enzyme-linked immunosorbent assay; IFA, immunofluorescent antibody.
[b]ELISA: OD492 value of 0.3 or higher was regarded as positive; IFA: Antibody titer 1:10 or higher was regarded as positive.

for *C. piliforme* in ELISA. Results also indicated that cross-reactions in ELISA could be differentiated by IFA. As long as a whole bacterial eluant is used as the antigen, it is difficult to avoid cross-reactions with other bacteria that have common antigens. For this reason, we use several methods concomitantly for each test item in antibody tests. From the previous ICLAS report, it appears that differences in the antigen strains used and in the testing systems caused the discrepancy in the results.

Hantavirus Testing by Serology

Hantavirus is a zoonotic agent for which rats are the reservoir. About 20 years ago, an outbreak of Hantavirus infection occurred in laboratory animal facilities in Japan. More than 100 animal caretakers and researchers were infected, and one died. Thereafter, thorough testing was undertaken, contaminated facilities were disinfected, and all animals were replaced. Contamination of animal experimentation facilities was eliminated in a short time. A description of our experience with antibody testing of this virus follows.

Antibody testing of this virus was possible in several facilities in Japan at the time of the outbreak. The ICLAS Monitoring Center sent technicians to Fort Detrick in the United States with an introduction from Dr. Allen, who is here today, and also to the testing facility for this virus in a university in Japan. We introduced an antibody testing technique using IFA, which currently, along with immunoblot and ELISA methods, is available.

Several years ago, we received a report from the university testing facility that rats in the laboratory animal facility of a medical school were Hantavirus

antibody positive. All animal experiments were immediately stopped and the animals destroyed. Later we were asked to test the samples, and the results were negative. Testing was performed using basically the same method except for a slight modification to inhibit nonspecific reactions; however, the results were different.

The results in the testing facility of the university were considered positive at an antibody titer of 1:16. They took the position of stating only that there was a positive reaction and deferring to the client to decide whether there was an infection, even though they were experts in this field. When we obtained results showing that antibody titers were in the 1:20 to 1:100 range (so-called low antibody titer) in the IFA test, we reported these results to the client while continuing the testing process. Inasmuch as there was little possibility of real infection, we collected blood samples after 1 week for retesting because generally in contaminated facilities, both the prevalence and antibody titer increase when retesting is performed. We have performed Hantavirus antibody tests on several thousand samples a year and have found only about 10 samples showing such low antibody titers. However, these samples were all found to be negative using immunoblot analysis.

The discrepancy between our test results and those of the university appeared to be caused by differences in the basic position of the testing facility when submitting the test results to the client. There are two different positions: One is simply to hand over the test results, and the other is to consider countermeasures after obtaining the results and assuring highly accurate test results. We naturally take the latter position.

CONCLUSION

The reasons for discrepancies in culture results include difficulty in accurate identification of isolates and differences in sample collection sites and tests for identification of bacteria. For the several test methods available in antibody tests, it is necessary to confirm the advantages and disadvantages of each method and select the method based on an overall evaluation of the factors. The position taken by the testing facility concerning its level of responsibility when submitting test results will also influence the results.

I am well aware that test methods are in a constant state of development, test facilities take pride in their techniques, and it is very difficult to achieve uniform test methods. I want to stress that simply listing the test items is not sufficient for international harmonization of microbiologic testing of laboratory animals. The test items must include the test method as well as a recommendation of usefulness. Even when there are no major differences in test methods, there are cases in which different results are presented to the client. These differences probably depend on the philosophy adopted by each testing facility.

Necessity of Reexamining the Pathogenicity and Elimination of Parasites in Rats and Mice

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MICROBIOLOGIC CONTAMINATION IN LABORATORY RATS AND MICE

Recently, the Japanese Association of Laboratory Animal Facilities of National Universities (JALAN; Figure 1) addressed the issue of a possible microbiologic contamination problem upon delivery of laboratory rats and mice to national universities and associated institutions. JALAN's Working Biosafety Committee (of which I am a member) has begun an investigation, and results have indicated that the problem is caused by the discrepancy between the sterility monitoring policies of the sender and the recipient. In some institutions, potentially harmful microbes/parasites (e.g., pinworms) are considered insignificant, and laboratory animals that are comtaminated with such microbes/parasites are sent or accepted without being examined. I would like to call attention to this problem and propose countermeasures.

Contamination of laboratory animals introduced both domestically and from abroad and the discrepancy between senders' and recipients' sterility policies were reported by Dr. Mannen (Oita Medical University, Japan) at the last US/Japan conference (Mannen 1998). Dr. Mannen stated that many members of JALAN encounter subtle differences between the required microbiologic monitoring/inspection items of animals being distributed among national, public, and private colleges, as well as other academic institutions, and that this type of problem also exists with international shipping. Problem cases are increasing particularly with the increase of transported gene-manipulated animals (e.g., transgenic/knockout mice). For this reason, the JALAN Biosafety Committee is

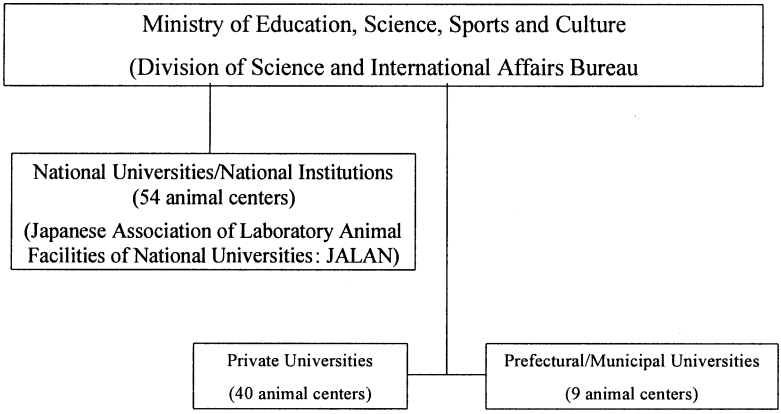

FIGURE 1 Organizational chart of animal centers in Japanese universities. Adapted from Mannen, K. 1998. Definition of microbiologic status of rats and mice: The need for methods of defining flora: International standards for terminology. In: Microbial and Phenotypic Definition of Rats and Mice: Proceedings of the 1998 US/Japan Conference. Washington DC: National Academy Press. p. 24–27.

dedicated to the difficult task of formulating guidelines on the delivery and acceptance of laboratory rats and mice in national universities in an effort to establish uniformity in microbiologic monitoring/inspection items.

The results of inspecting mice facilities at the Central Institute of Experimental Animals of Japan for parasites over 3 years are shown in Table 1. Parasites were detected in 125 of 444 facilities. Some of the parasites are nonpatho-

TABLE 1 Parasitologic Monitoring in Mice Experimental Facilities (1996-1998)

Parasites	Number of Positive Facilities (%)
Octomitus pulcher	41 (9.2)
Chilomastix spp.	18 (4.1)
Tritrichomonas spp.	18 (4.1)
Syphacia obvelata	14 (3.2)
Entamoeba muris	10 (2.3)
Pneumocystis carinii	9 (2.0)
Aspiculuris tetaptera	7 (1.6)
Spironucleus muris	3 (0.7)
Unknown protozoa spp.	5 (1.1)

genic; however, others (e.g., *Aspiculuris* and *Spironucleus*, from both domestic and international sources) are pathogenic.

Members of the Biosafety Committee have been reconsidering the pathogenicity of specific pathogens (Itoh 1998). They currently recommend monitoring for the pathogens listed in Table 2, with optional attention to nonpathogenic protozoa. Although Committee members discussed the possible deletion of nonpathogenic protozoa (e.g., trichomonads) from the list, because they are sometimes found and/or listed in health reports from outside a facility, they ultimately decided to retain the item in the list.

PINWORMS AS POSSIBLE INDICATORS OF BIOLOGICALLY CONTAMINATED ANIMAL FACILITIES

Some Committee members considered the presence of nonpathogenic protozoa to reflect the level of microbiologic control among animal facilities. In the case of pinworms, the ICLAS monitoring center has identified pinworms as category E (i.e., a nonpathogenic parasite and mere indicator). From my experience, I have no doubt that pinworms have caused diseases, have affected their physiologic functions, and have influenced experimental results. Reports of pinworm infection affecting experimental results include that of Wagner (1988), who reported definite growth differences between pinworm-free and pinworm-infected rats. Sato et al. (1995) also reported that antibodies against *Syphacia obvelata* somatic antigen were detected in experimental infection of pinworms in mice. Thus, it is clear that pinworms affect infected animals' physiologic functions and thereby influence experimental results. In my opinion, laboratory animal scientists should no longer minimize the influence of parasites. Because the measures taken to combat parasitic contamination in laboratory rats and mice have conspicuously lagged behind those taken against other pathogens (e.g., viruses and bacteria), parasites may currently be found even in animal facilities that appear to be well maintained and without microbiologic problems.

From an international point of view, I highly recommend international unification and harmonization of allowable microbiologic monitoring/inspection items of animals.

NEED FOR ELIMINATION OF PARASITES

In my experience with pinworm infection of laboratory mice, parasites have been entirely eliminated from a colony by mixing anthelmintic with feed and/or spraying the animals or bedding with ivermectin. Although these methods (especially the use of ivermectin) do involve some risk and are therefore not recommended, they are easier and more convenient than the embryo-transplant method. Some researchers complain about the use of anthelmintic; however, they should not overlook the spread of parasite infections like pinworms. I believe these methods are necessary to eradicate pinworms and other parasitic infections from mouse and rat colonies.

TABLE 2 Microbes/Parasites in Mice and Rats for the Subject of Microbiologic Monitoring in University Animal Facilities

Mouse Pathogen	Category[a]	Frequency[b]	Microbiological status[c]	Inspection
Mouse hepatitis virus	B	☆☆☆	Minimum	Periodically
Sendai virus	B	☆☆☆	Minimum	Periodically
Ectromelia virus	B		Minimum	Nonperiodically
Lymphocytic choriomeningitis virus	A		Minimum	Nonperiodically
Mouse rotavirus (EDIMV)	B/C	☆☆	Common	Nonperiodically
Mouse parovirus (MVM/MPV)	C	☆☆	Common	Nonperiodically
Mouse encephalomyelitis virus (TMEV)	C	☆☆	Common	Nonperiodically
Pneumonia virus of mice (PVM)	C	☆☆	Common	Nonperiodically
Mouse adenovirus	C	☆	Common	Nonperiodically
Reovirus type 3	C	☆	Common	Nonperiodically
Lactic dehydrogenase elevating virus (LDEV)	C	☆☆	Common	Nonperiodically
Mycoplasma pulmonis	B	☆☆☆	Minimum	Periodically
Salmonella spp.	A	☆	Minimum	Periodically
Clostridium piliforme (Tyzzer's organism)	C	☆☆	Minimum	Periodically
Corynebacterium kutscheri	C	☆☆	Minimum	Periodically
Pasteurella pneumotropica	C	☆☆☆	Common	Periodically
Cilia-associated respiratory (CAR) bacillus	C	☆☆	Common	Nonperiodically
Escherichia coli O115a, c:K(B)	B/C	☆	Common	Nonperiodically
Helicobacter hepaticus	C	☆☆	Common	Nonperiodically
Pseudomonas aeruginosa	D/E	☆☆☆	Excellent	Peri./nonperi.
Staphylococcus aureus	D/E	☆☆☆	Excellent	Periodically
Pneumocystis carinii	D	☆☆	Excellent	Nonperiodically
Pathogenic protozoa				
Giardia muris	C	☆☆	Common	Periodically[d]
Spironucleus muris	C	☆☆	Common	Periodically[d]
Nonpathogenic protozoa				
Trichomonads, etc.	E	☆☆☆	Excellent	Periodically[d]
Helminths (pinworms)	C	☆☆☆	Common	Periodically[d]

Rat Pathogen	Category[a]	Frequency[b]	Microbiological status[c]	Inspection
Sialodacryoadenitis virus (SDAV)	B	☆☆☆	Minimum	Periodically
Sendai virus (HVJ)	B	☆☆☆	Minimum	Periodically
Hantavirus	A	☆	Minimum	Periodically
Rat parvovirus (KRV/H-1/RPV)	C	☆☆	Common	Nonperiodically
Mouse encephalomyelitis virus (TMEV)	C	☆	Common	Nonperiodically
Pneumonia virus of mice (PVM)	C	☆	Common	Nonperiodically
Mouse adenovirus	C	☆	Common	Nonperiodically
Reovirus type 3	C	☆	Common	Nonperiodically
Mycoplasma pulmonis	B	☆☆☆	Minimum	Periodically
Salmonella spp.	A	☆	Minimum	Periodically
Clostridium piliforme (Tyzzer's organism)	C	☆☆	Minimum	Periodically
Corynebacterium kutscheri	C	☆☆	Minimum	Periodically
Bordetella bronchiseptica	C	☆☆	Minimum	Periodically
Pasteurella pneumotropica	C	☆☆☆	Common	Periodically
Streptococcus pneumoniae	C	☆	Common	Nonperiodically
Cilia-associated respiratory (CAR) bacillus	C	☆☆	Common	Nonperiodically
Pseudomonas aeruginosa	D/E	☆☆☆	Excellent	Peri./nonperi.
Staphylococcus aureus	D/E	☆☆☆	Excellent	Nonperiodically
Pneumocystis carinii	D	☆☆	Excellent	Nonperiodically
Pathogenic protozoa				
Giardia muris	C	☆☆	Common	Periodically[d]
Spironucleus muris	C	☆☆	Common	Periodically[d]
Nonpathogenic protozoa				
Trichomonads, etc.	E	☆☆	Excellent	Periodically[d]
Helminths (pinworms)	C	☆☆	Common	Periodically[d]

[a]Criteria used for selection of test items in the ICLAS monitoring center.
[b]Frequency of occurrence during the past two decades in Japan. ☆, rare; ☆☆, often; ☆☆☆, very often.
[c]Minimum: These pathogens should be negative. Common: It is desirable that these pathogens are negative. Excellent: It is desirable that these pathogens are negative in immuno-insufficient/deficient mice.
[d]Inspection methods to be recommended are to examine small/large intestine contents.
(Modified in part from Mannen, 1998)

CONCLUSION AND RECOMMENDATIONS

- Measures to counteract parasitic contamination in experimental animals, especially rats and mice, lag conspicuously behind those that counteract other pathogens.
- Parasites often serve as indicators of the level of microbiologic control among animal facilities. However, because some parasites may affect experimental data, their existence and species name should be clearly indicated in the health monitoring reports.
- The pathogenecity of parasites should be reexamined, even if they are considered nonpathogenic to the animals. An international scheme for unification/harmonization of test results and allowable conditions should be devised.
- Parasites should be eliminated as much as possible. The methods recommended are as follows:
    1. mixing anthelminthic with feed;
    2. spraying animals/bedding with anthelminthic; and/or
    3. cleaning the colony using a method such as embryo transplant.

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Emerging (and Reemerging) Viruses of Laboratory Mice and Rats

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INTRODUCTION

Results of serologic tests performed to monitor laboratory rodents for infectious diseases are dramatically more accurate now than in the 1970s when serologic testing was largely limited to complement fixation and hemagglutination inhibition (HAI) tests. It was, in fact, the improvements in diagnostic testing that led to our appreciation of some "emerging" infections. The new generation of tests also revealed unexpectedly high prevalence of infection with some agents we thought were present at low levels in commercial and academic facilities. For example, many sera from mice that had sustained infection with mouse hepatitis virus yielded uninterpretable (anticomplementary) results in the complement fixation test. These were reported as "unsatisfactory" but were clearly positive when tested retrospectively by indirect fluorescent antibody (IFA) tests and enzyme immunoassays.

The agents discussed here were chosen for several reasons. They include (1) prevalence; (2) documented interference with biomedical research; (3) difficulty in eliminating and/or preventing infection, especially in populations of genetically altered rodents housed under crowded conditions; and (4) periodic reemergence from unexpected sources.

AGENTS OF CONCERN

Mouse Parvoviruses

The existence of at least one previously unrecognized parvovirus of mice was suspected in the early 1980s when the HAI assay was replaced in some laboratories by IFA tests that used minute virus of mice (MVM) as antigen. A

proportion of mouse sera that clearly reacted with MVM antigen in the IFA test yielded negative results in the HAI assay that was used for confirmation. Although some opined that the IFA test simply yielded false-positive reactions, others were suspicious that there was an agent (or agents) distinct from MVM circulating in laboratory mouse colonies. As we know, the latter was the case, and the discrepant results of the two tests were based on the fact that the IFA test permitted recognition of both structural and nonstructural proteins of parvoviruses. The nonstructural coding regions of MVM and the newly recognized mouse parvovirus (MPV) are essentially identical, whereas the structural regions (recognized by sera in the HAI assay) are quite divergent. It is likely that those differences in the structural region account for the fact that the humoral immune response protects only against homotypic parvovirus infections of mice (Hansen and others 1999).

The existence of a putative new parvovirus was supported by studies at Yale, where transmission within an enzootically infected breeding colony of mice was documented. Medium from cultured peripheral blood lymphocytes and explanted spleens of seropositive mice contained a substance that agglutinated mouse erythrocytes; however, the hemagglutination could not be inhibited by antibody to MVM, rat virus (RV), or H-1 virus (A.L. Smith, unpublished data). Several years later, a cellular immunology laboratory at the University of Chicago began to have difficulty maintaining cloned T cell lines. Some cell lines died suddenly, and others simply failed to thrive. The presence of aggregated mouse erythrocytes was perceived to be a reliable indicator of infection with a putative virus (McKisic and others 1993). Southern blot analysis revealed the presence of a parvovirus that was shown serologically to be distinct from both the prototype and immunosuppressive allotropic variants of MVM. Infected cultures responded poorly to specific antigen and to interleukin 2. The agent was presumably introduced into the laboratory by spleen cells used as feeders and/or substrates for producing growth factors in mixed lymphocyte cultures. That laboratory, as well as others concentrating on murine T cell immunology, had episodic difficulty maintaining T cell lines and clones. This may have been due to incomplete decontamination after infections were recognized—parvoviruses are notoriously stable in the environment. Retrospective serology confirmed that MPV has circulated in US mouse colonies at relatively high prevalence for more than 25 years (Jacoby and others 1996).

Mouse parvovirus has an ideal relationship with its natural host: Infected mice of all genotypes and ages so far tested remain clinically normal and manifest no pathologic changes. The virus does not follow all parvoviral dogma—for instance, adult mice are at least as susceptible as neonates to MPV infection (Smith and others 1993). This is in contrast to the situation with MVM and most other parvoviruses. The generally higher susceptibility of neonates is likely attributable to the requirement by parvoviruses of a cellular factor present during S phase for their own replication.

In situ hybridization studies with tissues from experimentally infected mice have revealed that MPV replicates preferentially in the small intestine and in lymphoid tissues. Viral DNA is apparently cleared from the intestine at some time after seroconversion but can be detected in lymphoid organs of experimentally infected mice for at least 9 weeks (Jacoby and others 1995). A single study has evaluated the serologic and virologic characteristics of mice from a colony enzootically infected with MPV (Shek and others 1998). Two-, 3- and 6-month-old BALB/c mice from that colony had MPV DNA in lymph nodes, spleen, and small intestine. Infectious virus was recovered from the same tissues of some of those mice. One-month-old mice from the colony were seropositive, and a homogenate of pooled small intestine contained infectious virus. Only the 1-month-old mice transmitted infection to cage contacts. The implication is that mice in an enzootically infected colony remain infected for at least 6 months and possibly for life; however, transmission studies suggested that older mice do not transmit infection to cage contacts, a small consolation for colony managers. We do not yet know whether intestinal infection can be reactivated by environmental factors or experimental manipulation, resulting in recurrent transmission.

The persistence of MPV in lymphoid tissue raised the possibility of aberrant or inappropriate immune responses against antigens to which the host might be exposed. Studies designed to address that possibility have revealed that MPV infection does modulate T cell effector function. Tumor allografts were rejected at an accelerated rate by MPV-infected mice, and T cells from infected mice that had rejected the tumors had diminished cytolytic capacity (McKisic and others 1995). In a separate series of studies, MPV potentiated the rejection of allogeneic skin grafts, but proliferation of alloantigen-reactive lymphocytes from graft-sensitized mice was reduced. Unexpectedly, MPV also induced rejection of syngeneic skin grafts, and T cells from infected, graft-sensitized mice lysed syngeneic target cells (McKisic and others 1998). Autoimmunity as a consequence of MPV infection is intriguing in view of recent reports suggesting that B19 virus may induce autoimmune disease in humans (Lunardi and others 1998; Vigeant and others 1994).

Ideally, biologic contaminant testing should be done on any cultured cells or tumors destined for use in laboratory animals. The policy is difficult to enforce, and some investigators must be convinced that compliance is in their best interest. Cells that will be injected into mice or rats must always be tested for rodent parvoviruses because of their affinity for rapidly dividing cells, such as tumor cells or lymphocytes. Additionally, there have been several instances in recent years of MVM infection of cells in large-scale production bioreactors (Chang and others 1997; Garnick 1996; A.L. Smith, unpublished data). Several laboratories have developed polymerase chain reaction methods for detection of rodent parvovirus contamination (Besselsen 1998; Besselsen and others 1995; Chang and others 1997; Garnick 1996; Riley and others 1999). This sensitive, rapid methodology should improve investigator compliance with institutional testing policies

because results can be available within 24 hours of sample submission. This contrasts with the 3- to 6-week turnaround interval for mouse (or rat) antibody production tests.

Rat Parvoviruses

Rats are now known to harbor multiple parvoviruses. These agents seem to display more genetic heterogeneity than do parvoviruses of mice; however, this could simply be due to the fact that more rat parvoviruses have been isolated and characterized. The virus now called rat parvovirus (RPV)-1a shared only 82% amino acid identity in the NS coding region with the UMass strain of RV (Ball-Goodrich and others 1998). Four additional rat parvovirus isolates, two from wild rats, have been characterized at the molecular level. The two isolates from wild rats (RPV-2a) were identical, and there was 95% protein sequence similarity among those two viruses and two others (RPV-2b and RPV-2c) from geographically distinct colonies of laboratory rats (Wan and others 1999). However, nucleotide homology of the RPV-2a isolates, RPV-2b and RPV-2c, with the isolate characterized by Ball-Goodrich and others (1998) was only 73%.

A recent serologic study indicated that the prevalence of RPV in rats in Japan ranged from 13 to 22% (Ueno and others 1998). The same investigators reported that an uncharacterized isolate of RPV preferentially infected lymphoid tissue and was excreted in feces, urine and nasopharyngeal secretions (Ueno and others 1997). Viral DNA could be detected in lymphoid tissues for at least 24 weeks. RPV-1a preferentially infects lymphoid tissues and endothelium and, unlike isolates of RV, is enterotropic as well (Ball-Goodrich and others 1998).

Based on their affinity for lymphoid tissues, rat parvoviruses might be expected to modulate immune responses. The Kilham strain of RV induced T cell-dependent autoimmune diabetes in several strains of rats (Ellerman and others 1996). The UMass strain of RV infects both $CD4^+$ and $CD8^+$ T cells as well as B cells (McKisic and others 1995). T cells from infected rats proliferated poorly and had reduced cytolytic capacity. Nonlethal infection of a $CD4^+$ T cell line resulted in reduced proliferation in response to antigen and interleukin 2 (McKisic and others 1995). The immunomodulatory properties of RPV isolates have not yet been explored.

Mouse Hepatitis Virus (MHV)

Mouse hepatitis virus is, in a sense, reemerging. The virus was successfully eliminated from many vivaria during the last 10 to 15 years. This was facilitated by the knowledge that infection is acute and self-limiting (Barthold and Smith 1987) and that cessation of breeding for a period of a few weeks would permit infection within a room to burn out (Weir and others 1987). Today we appear to be in the midst of a national epizootic of MHV, and recent finger pointing has

shed more heat than light on the problem. Why do we find ourselves in this frustrating situation? What follows is partly speculative and partly based on observations of vivaria at several large universities. First, it is almost impossible to eliminate MHV from every niche in an academic setting. There will always be one influential investigator who *absolutely cannot, under any circumstances,* stop his/her experiments long enough for the infection to run its course. If there is a single room, no matter how remote, housing MHV-infected mice, the risk of transmission exists. That risk can be traced to HVAC systems, fomites, and human tracking, among other possibilities. Yet influential investigators, the malfunctioning HVAC systems, fomites, and people who enter rooms out of order have always existed. What is so different now? The answer lies, I believe, in the nature of the contemporary murine host and the population density of that host. Genetically altered rodents are being developed and used in biomedical research at a staggering rate. Many of these animals sustain MHV infections that appear to be of longer duration than that seen in "normal" mice. In addition, it is not at all unusual to find mice housed at such high density that the filter tops, which formerly afforded protection against infections, are tipped and provide no physical barrier at all. Cage and rack manufacturers are responding to the problem with innovative designs for ventilation and maintenance of larger numbers of cages per rack. However, academic institutions are slow to respond with the resources needed to fix the problem on a more permanent basis—new facilities. It is probably not unreasonable to predict that any facility currently in the design phase will be filled to capacity on the opening day.

The risks to research done with MHV-infected mice have been well documented. MHV infection modulates both T cell and antigen-presenting cell function (de Souza and Smith 1991; de Souza and others 1991; Smith and others 1991). B cells of genetically susceptible strains of mice may become infected but remain viable and morphologically normal (de Souza and Smith 1991). More recent reports have shown that MHV can also noncytolytically infect embryonic stem cell lines derived from mice of many genetic backgrounds (Kyuwa 1997). This finding makes it imperative to use feeder cells from uninfected mice. Infected embryonic stem cells apparently do express normal levels of differentiation markers (Okumura and others 1996).

Ectromelia Virus

The causative agent of mousepox reemerges in the United States once or twice each decade, and it is included here because we need to be reminded periodically that this can happen. Earlier this year ectromelia virus was introduced into a single mouse room at Weill Medical College of Cornell University (Lipman and others 1999). The source of the infection was pooled mouse serum that had been prepared in China as a batch of at least 43 liters in early 1995. Given the volume, it is highly likely that there will be additional outbreaks

associated with this lot of serum. A similar outbreak occurred in 1995 at the Naval Medical Research Institute in Bethesda, Maryland (Dick and others 1996). Again, a single room was involved. Clinical signs were mild, the most common being conjunctivitis, and mortality was low. The source of infection was pooled mouse serum that had been prepared within the continental United States. The reagent was marketed for use in culture medium, not in vivo use. However, it is not unusual for cultured cells of several types (which may, themselves, be free of contaminating agents), grown in media supplemented with animal serum, to be injected into laboratory rodents. What is most surprising is that this was the *sole* outbreak to arise from use of that batch of pooled mouse serum. However, Jacoby and Lindsey (1998) reported that some respondents to a survey for infectious diseases in US rodent colonies indicated the presence of ectromelia virus infection in non-specific pathogen-free colonies of mice.

CONCLUSIONS

It is unlikely that any of the agents discussed here will be eradicated in the foreseeable future. In fact, several may increase in prevalence as more diverse transgenic and knock-out animals are developed. That some of these animals may have immune dysfunction is not always predictable based on the targeted genetic change. Compounding the problem is the issue of housing density, which contributes to transmission of infections among animals just as it does in human institutions such as day-care centers. Perhaps the best analogy in humans is adenovirus-associated acute respiratory disease in newly assembled military recruits housed under crowded conditions.

Will new or newly recognized infectious agents emerge among laboratory rodents? The answer is, emphatically, yes. This emergence is predicted by recent experience: Several rodent parvoviruses and *Helicobacter* species have been recognized in a relatively short span of time. Riley and colleagues have presented evidence of an infectious etiology for idiopathic lung lesions in rats (Riley and others 1999). Unquestionably, there will be more agents to keep veterinary microbiologists and virologists gainfully employed!

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Emerging Infections as a Cause of Concern

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In a very informal sense, emerging infections are the unexpected—those infections that appear unexpectedly without warning and often rapidly. In a more formal sense, they are infections that rapidly expand in geographic range or in prevalence or that appear suddenly in a population. In the case of laboratory animal colonies in the United States and Japan, the level of infectious diseases has been decreasing over the years.

IMPERFECTLY CONTROLLED ENVIRONMENT

The laboratory animal environment is among the best controlled in the world. Although there are still obvious imperfections, as we heard from Dr. Smith (this volume), they are relatively well controlled. We have seen many infections such as ectromelia become comparatively rare; however, such improvements should also give us cause to maintain our guard—to remember that these infections have not been eradicated. Even smallpox, which was officially eradicated in a great cooperative venture (for which Japanese scientist Esau Arita and others deserve a great share of the credit), is something we argue about as a potential bioterrorist threat. Smallpox is the only infection that has successfully been eradicated even among human beings, and the opportunity exists for any infection to remain in geographically isolated colonies. There are places in the world where ectromelia still exists in rodent colonies, and Dr. Riley has described the traffic and movement of laboratory animals and their products throughout the world, which is one of the many factors that could allow a localized infection to spread if we are not vigilant.

In addition to opportunism, there is risk associated with the vast biodiversity of microorganisms that can be introduced into a laboratory animal colony through

contact with the natural host in the environment. In this respect, hantaviruses are of particular interest. The entire concern about emerging infections actually originated in a very small way about 10 years ago from a question about whether hantaviruses were a risk to laboratory workers. At that time, we knew of hantaviruses as an occupational hazard. In recent years, many more have been discovered, and now we know of literally dozens throughout the world, including a great number discovered only in the last few years throughout North and South America where they are harbored in very common wild rodents. Of course the possibility exists for these new infections to be introduced in facilities where other animals from the outside are brought into contact. Such knowledge of the great, not yet fully tapped biodiversity of microorganisms requires us to avoid complacency and to understand that detection and diagnosis remain essential tools for control.

RECOGNIZED THREATS

Why might we be interested or concerned about emerging infections in laboratory animals? One compelling reason for all of us as laboratory animal disease specialists is of course the potential threat to the colonies as described by Dr. Smith and other speakers. The possibility exists that ectromelia could be reintroduced, and it is always a potential danger; many examples of rodent parvoviruses are a cause for concern, and perhaps the most dramatic parvovirus was canine parvovirus 2, which appeared in the late 1970s in dogs. There are still many discussions about how it was introduced, but it may have been an accident in vaccine production, a contaminant by another parvovirus during vaccine. We know from work by Colin Parish and others that once this virus appeared, it was able through essentially one mutation to expand its host range into dogs. It spread rapidly throughout the dog population causing very high mortality in puppies and other rapidly growing young dogs. It was replaced by another variant, and several waves of this process took place until several strains of this virus were distributed throughout the world.

Another emerging infection example is that of callitrichid hepatitis, which was really lymphocytic choriomeningitis (LCM), introduced into tamarins in the zoo. There it spread rapidly with rather fatal results throughout the captive tamarin population, possibly due to the feeding of contaminated material from newborn mice that had not been tested for LCM. The progression of this infection and others indicates the potential for surprise with a great biodiversity of microorganisms in existence.

OPPORTUNITIES TO LEARN

Nevertheless, we should not despair over emerging infections. We know there are factors responsible for emerging infections introduced into a new popu-

lation. We have heard that with some methods (such as crossing species), material may be introduced that may be infected from another species or contact with natural hosts through changes. That material may carry previously unfamiliar but possibly severe infections simply by chance through contact with natural hosts that are carrying these infections (such as the hantaviruses).

Another reason for laboratory animal specialists not to despair is related to the many opportunities in comparative medicine as well as is identifying and studying appropriate animal models. Understanding the pathogenesis of infectious disease in many cases is greatly facilitated by having a good animal model. Indeed, as you know, good animal models often are essential.

Many of our problems in understanding the pathogenesis of, for example, AIDS and HIV infection stem from the fact that we have a great limitation in animal models. In many other infections, a good small animal model would greatly facilitate our understanding of the host-pathogen interaction and our ability to study the natural process of disease and its pathogenesis in a relatively realistic situation. In addition, many zoonotic infections found in rodents and other animals may enable us to understand population dynamics through natural study, study in the laboratory, and comparative study of laboratory animal models. Similarly, there are pathogens not known to be human pathogens that offer interesting opportunities for study because of their existence in laboratory animals. Endogenous retroviruses are examples in that mice and rats have many known murine leukemia-like sequences that at times and under certain circumstances reactivate to give a variety of infective retroviruses, which we do not see in humans. I believe this model will provide interesting insights into some of the threats we may face in the future.

Finally and very importantly is the opportunity to study chronic disease models, often perhaps in their natural or more nearly natural setting that may also closely resemble the human situation. *Helicobacter* is one example of such a model, and I suspect there are many other chronic disease models that can be found and demonstrated coming from our knowledge about existing infections or those newly introduced in laboratory animal colonies. I will defer to Dr. Fox who has done much outstanding work on that subject.

PAST SURPRISES

Similarly, we are sometimes surprised by what we find in laboratory animals. Some years ago we studied a virus called mouse thymic virus (murine herpesvirus 3), which had the interesting property of destroying CD4-positive cells. Recently we collaborated with Drs. Shimo Shakabutshi and Noriko Shakabutshi, who were able to demonstrate and publish in the *Journal of Immunology* the regeneration of the thymus after these animals had been infected and had actually recovered while shedding virus for most of their lifetime. Their thymuses regenerated; however, often late in life, they would manifest disease,

indicating that disregulation of T cell differentiation could be an underlying mechanism for autoimmunity and possibly other abnormalities. This process was first observed with a fairly mild natural infection of mice. Human herpesvirus 6, which was discovered later, has many properties similar to murine herpesvirus 3 (mouse thymic virus), including its ability to kill T lymphocytes.

FUTURE DISCOVERIES

Thus, I believe there are many discoveries yet to be made. In addition, given the vast biodiversity of microorganisms and the geographic distribution of animal colonies all over the world, I fear there are possibilities for new, currently unknown infections to emerge. Some may be zoonotic and others (simply, like the examples described, but perhaps not so simply) may be capable of causing serious concern in laboratory animal colonies but either damaging the productivity of the colony or affecting the results. How we deal with these possibilities is the subject of this conference, and I believe the approaches are entirely appropriate. They remain our first line of defense: detection and diagnostics, the identification of disease organisms, that ever-increasing list of ever-dwindling organisms. In addition, it might also be advisable to have some broad, perhaps more generic, strategies for looking at pathogen discovery, such as generic polymerase chain reaction or differential display methods that would more rapidly identify pathogens present in the population for which we cannot yet test.

Briefly, gene expression and host markers also offer interesting possibilities. We now have the technology to look at gene expression in the host in response to pathogens, which may be markers of disease. They may also tell us a great deal about the host-pathogen interaction during experimental studies.

The question of validation has been posed many times, and I believe it remains an essential question with any diagnostic test. I also believe there is much cause for optimism in that context because a number of groups in these areas are cooperating, which I hope will continue along with an exchange of information and validation of reagents and tests.

DARPA

As a program manager at the Defense Advance Research Projects Agency (DARPA), I could not of course participate in this conference without commenting on DARPA. DARPA is best known for having originated the Internet, then called the ARPAnet (we were then called ARPA, a few years ago). Since that time, DARPA's mission has been to develop new technologies for critical national needs.

A few years ago, our director became particularly concerned about our vulnerability to biowarfare and bioterrorism. The decision was made to take a very broad approach to the diagnosis and identification of pathogens and to dealing

with host-pathogen interaction because although we talk a great deal about lists, the fact to remember is that although lists are a useful place to start, they are not the end point.

In any case, we do have a diagnostics program about which I welcome your comments and questions. For more information about DARPA, please feel free to browse our Web site (<http://www.darpa.mil/DSO/rd/Abmt/Bwd.html>). We are trying to develop new technologies and new approaches for the identification of infection and infectious pathogens that we hope will be useful in the future.

Emerging Diseases in Mice and Rats

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The two topics of my discussion are examples. The first topic is an example of contamination of a human tumor line passaged in immunodeficient mice, which illustrates the effects of *Helicobacter hepaticus* infection on animal experiments. The second topic is not actually related to *H. hepaticus* infection, but I use this organism to illustrate a method for establishing test items when a new infection appears in laboratory animals.

H. HEPATICUS CONTAMINATION OF HUMAN TUMOR TISSUES PASSAGED IN IMMUNODIFICIENT MICE

One aspect of cancer research at the Central Institute for Experimental Animals involves the collection of tumor tissues from patients and the subcutaneous inoculation into immunodeficient animals such as nude mice or mice with severe combined immunodeficiency (SCID) mice. In this process of passaging human tumor tissues using immunodeficient mice, focal necrosis appears very often in the liver of the cancer-bearing mice after a certain period. I describe below the characteristics of the infected mice, the process of the infection, and the countermeasures against it.

Although bacteriologic and histopathologic tests were performed using mice with liver lesions, and serum antibody tests were performed using sentinel mice to detect known pathogens, the cause of the lesions was not clear at the start of testing. After *H. hepaticus* was recognized as a new mouse pathogen and after a test method was established in our laboratory, it was confirmed that the cause of this abnormality was infection with this organism. According to results of tests

on the cancer-bearing mice, the lesions were the same as those reported previously in immunodeficient mice, that is, focal liver necrosis and proliferative colitis. Curved bacilli were detected in the bile canaliculi surrounded by necrosis and in the crypts of the large intestine.

The specific DNA sequence of this organism was detected by polymerase chain reaction (PCR) in livers (20/21), cecal contents (21/21), and transplanted tumors (19/20) in these cancer-bearing mice. It was assumed that *H. hepaticus* bacteremia occurred in the infected immunodeficient mice similarly to the occurrence of *Helicobacter* bacteremia in AIDS patients. We first determined when *H. hepaticus* was introduced into the colony. PCR for *H. hepaticus* in cryopreserved tumors strongly suggested that the contamination occurred in 1990, as shown in Table 1. Based on the time of the tumor contamination and facility records, we suspect that this organism was brought into the facility by genetically engineered mice introduced from overseas.

We then investigated eliminating this organism from the contaminated tumor tissues. We passaged the tumor tissues collected from these infected animals directly into noncontaminated SCID mice under sterile conditions; however, after several weeks, we found that the organs and tumors of the mice were *H. hepaticus* DNA positive. In subsequent investigations, after cryopreserved samples of *H. hepaticus* DNA-positive tumor tissue were thawed and transplanted into SCID mice, we found that these mice were *H. hepaticus* free, and this organism had also been eliminated from the transplanted tumor tissues. From these results, it was assumed that the SCID mice had bacteremia due to *H. hepaticus* infection and the transplanted tissue was also contaminated. However, the bacterial count in each cryopreserved sample was low, the bacteria in the tissue were killed by the

TABLE 1 *Helicobacter hepaticus* Contamination in Cryopreserved Human Tumor Xenografts in Immunodeficient Mice

Year of Cryopreservation	No. Examined	No. Positive
1985	6	0
1986	18	0
1987	9	0
1988	5	0
1989	4	0
1990	12	1
1991	7	0
1992	20	1
1993	27	5
1994	11	1
1995	5	0

freeze-thawing process, and no infectious bacteria remained even though the tissue was contaminated. After that, we confirmed that the process caused a decrease in the viable number of *H. hepaticus* to 10^{-1} or 10^{-2}.

We found that in transplantation studies of tumors and organs using immunodeficient mice, *H. hepaticus* also caused persistent bacteremia in the same way that mouse hepatitis virus, Sendai virus, or *Mycoplasma pulmonis* infection causes viremia or bacteremia. As a result, the tumor and organ samples were contaminated. *H. hepaticus* should be considered one of the organisms that requires precautions in animal experimentation facilities. I would like to emphasize the importance of quarantine for biomaterials from immunodeficient animals.

ESTABLISHMENT OF TEST AGENTS

In the establishment of test agents for microbiologic monitoring, the issue is whether to establish individual test agents for each species (such as *H. hepaticus* or *bilis*) or to establish the tests for the genus as a whole (such as *Helicobacter* spp.). According to the FELASA recommendations, tests for *Pasteurella* spp. are specified for mice and rats, but tests for individual species (such as *Pasteuella pneumotropica* and *multocida*) are not specified. Several species of *Helicobacter* have been detected in mice, and some of these have not been confirmed as pathogenic. However, it appears that testing for *Helicobacter* spp. will be adopted in Europe and the United States. Therefore, I would like to present my thoughts concerning the selection of test agents with respect to international harmonization of quality control of laboratory animals.

I propose that organisms subject to testing in the microbiologic monitoring of laboratory animals be assigned a significance and be divided into the five main categories described in the *Manual of Microbiologic Monitoring of Laboratory Animals*, published as a result of prior US-Japan Meetings (USPHS/NIH 1994). These categories are A: zoonotic and human pathogens carried by animals; B: pathogens fatal to animals; C: pathogens not fatal but that can cause diseases in animals and affect their physiologic functions; D: opportunistic pathogens for animals; and E: indicators of the microbiologic status of an animal or colony.

At the ICLAS Monitoring Center, we believe that test agents should be selected for definite reasons. We therefore have established the following list for obtaining necessary information: whether the organism has been confirmed to be pathogenic, and effects on the experimental results are clear; convenience of testing (whether ordinary test methods are established and kits are available); prevalence or usefulness as an indicator of microbiologic control. An overly long list of test agents will result in an increase in costs over the whole range from production to the use of laboratory animals. Continuing qualitative improvement of laboratory animals cannot be expected with such an increase in costs. With respect to animal experimentation facilities in particular, the problem is how many users actually have facilities that can utilize such sophisticated animals.

In the harmonization of quality tests, it is clear that selection of test agents is the most important factor. Therefore, test agents adopted for the genus should not be increased aimlessly, and caution is required. Overtesting is of benefit only to testing facilities and some large-scale producers; it does not necessarily improve the quality of laboratory animals and animal experimentation, which is our objective. In the selection of test agents, I believe that additional consultation is necessary among researchers performing animal experiments, animal breeders, and testing facilities.

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Survey of *Helicobacter* Species in Laboratory Mice and Gerbils in Japan

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Since *Helicobacter muridarum* was first reported (Lee and others 1992), additional *Helicobacter* species have been isolated from intestinal tracts of rodents. In these subsequent studies, species such as *Helicobacter hepaticus* (Fox and others 1994), *H. muridarum*, *Helicobacter bilis* (Fox and others 1995), *Helicobacter rodentium* (Shen and others 1997) and "*Flexispira rappini*" (Schauer and others 1993) were surveyed using reverse transcription-nested polymerase chain reaction (PCR) to clarify the so-called current status of *Helicobacter* infection in laboratory mice and gerbils in Japan.

MATERIALS AND METHODS

For *H. hepaticus* detection, a total of 310 mice were sampled. The total included 116 mice from two breeding colonies, 194 mice from 19 research institutions, and 74 gerbils including 11 gerbils from two breeding facilities and 63 gerbils from six research institutions. RNA from the samples was transcribed to cDNA using *Helicobacter* genus-specific primers. Primers used for the first PCR were also *Helicobacter* genus specific. Primers for the second (nested) PCR were specific for *H. hepaticus*. *H. hepaticus*-specific primers were selected according to the previous report (Battles and others 1995).

For *Helicobacter* species detection, 149 mice from 17 facilities were used. The samples were different from those used in the *H. hepaticus* study described above. RNA was transcribed to cDNA using *Helicobacter* genus-specific primers, and primers for the first and second PCR were also *Helicobacter* genus specific. Using samples found to be positive with *Helicobacter* genus-specific

primers, the first PCR products were amplified with five sets of species-specific primers to identify *Helicobacter* species. For samples that could not be identified using the primer sets, the first PCR products were sequenced. To avoid nonspecific bands, the restriction pattern was evaluated.

RESULTS

Results of *H. hepaticus* detection are shown in Table 1. For mice, all 116 samples from two breeding facilities were negative, but 35 samples from five research institutions of the 194 samples from 19 research institutions were positive. For gerbils, seven samples from one breeding facility of the 11 samples from two breeding facilities were positive, and 43 samples from four research institutions of the 63 samples from six research institutions were positive. Multiple pale to yellow foci were seen on the liver surface of almost 50% of *H. hepaticus*-PCR-positive mice and gerbils.

Results of *Helicobacter* species detection are shown in Table 2. Seventy-nine of 149 samples were positive with *Helicobacter* genus-specific primers. Among the 79 samples, 20, 35, and 26 samples were identified as *H. hepaticus*, *H. rodentium*, and other species, respectively. Two samples from one facility were positive for both *H. hepaticus* and *H. rodentium*. No gross lesions were observed in the mice. According to the sequences of the first PCR products from the 26 samples classified as others, these samples were identified as *Helicobacter* species belonging to the same cluster as *H. rodentium*.

CONCLUSION

H. hepaticus was detected not only from mice but also from gerbils. In this study, it was suggested that the gerbil is one of the hosts of *H. hepaticus* infection.

TABLE 1 Detection of *Helicobacter hepaticus* 16S rRNA in Laboratory Mice and Gerbils Using Polymerase Chain Reaction

	No. Positive / No. Tested in Samples		No. Positive / No. Tested in Facilities	
Mice				
Breeding facilities	0 / 116	(0%)	0 / 2	(0%)
Research institutions	35 / 194	(18.0%)	5 / 19	(26.3%)
Gerbils				
Breeding facilities	7 / 11	(63.6%)	1 / 2	(0%)
Research institutions	43 / 63	(68.3%)	4 / 6	(66.7%)

TABLE 2 Detection of *Helicobacter* Species 16S rRNA in Laboratory Mice Using Polymerase Chain Reaction

Species	No. Positive / No. Tested in Samples		No. Positive / No. Tested in Facilities	
H. hepaticus	20 / 149	(13.4%)	1 / 17	(5.9%)
H. muridarum	0 / 149	(0%)	0 / 17	(0%)
H. bilis	0 / 149	(0%)	0 / 17	(0%)
H. rodentium	35 / 149	(23.5%)	6 / 17	(35.3%)
"F. rappini"	0 / 149	(0%)	0 / 17	(0%)
Others	26 / 149	(17.4%)	3 / 17	(17.6%)
Total	79 / 149	(53.0%)	10 / 17	(58.8%)

Because gerbils can be infected with *H. pylori* (Hirayama and others 1996), they are widely used for *H. pylori* infection studies. These results indicate the necessity of checking *H. hepaticus* contamination in gerbils. *H. hepaticus* and *H. rodentium* were the most common *Helicobacter* species in mice in Japan, and *H. muridarum*, *H. bilis*, and "*F. rappini*" were not detected.

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Genetic Evaluation of Outbred Rats

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The US Food and Drug Administration (FDA) comprises a very diverse group in Foods and Biologics and Veterinary Medicine and Drugs. We all have very different views on how animals should be used, in what kinds of studies they should be used, and how we use information from studies in assessing risk. My remarks are from the perspective of the Center for Drug Evaluation and Research and not from the perspective of any of the other centers within FDA.

REGULATORY PERSPECTIVE AND INFORMATION

Genetic marker information is not required in study protocols. It is not submitted as part of data analyses, even for explanations of deviations in study results. As an example, when I surveyed many of the reviewers within the Center for Drug Evaluation and Research about how often they received genetic profiles of the test animals, none had ever seen such information.

Industry simply does not provide it.

The information we typically receive is *required* information: strain identification, the source of the animals, and sometimes site-specific control data sets, which we may request in an effort to understand the significance of some finding because we do not have the control data of all sponsors or contractors. We do have complete control data, including line listings, from the animals on the study.

For example, if we have a particular question about vasculitis either in your breeding colony or from your source, we may request the historical response rate so that we can calculate whether there is a significant effect of the pharmaceutical in relation to variable background rates. Another very recent example involves

our carcinogenicity assessment committee, which evaluates all carcinogenicity studies that come into the Center for study conduct and results review, including a report received last week from an international company that had an unlicensed product. For their dose range finding study, the available general toxicology studies had been done in Japan, and the carcinogenicity study had been done in the United States. For their animals, they had gone to the same global supplier, which had two different colonies of animals—one in Japan and one in the United States. In the Japanese studies that were submitted, there was a phenotypic response in the animals to the drug that was unmistakable. Females lost about 100 g/kg or up to 20% of their body weight. Males were not affected. Thus, the effect that occurred in the dose range finding studies at 3 months (and later, in a separate study, at 6 months) were persistent and obviously drug related. However, in the carcinogenicity study, with the same dose levels as in the other studies and with animals from the US source, there was no effect on body weight at any time during the study.

The question is whether this difference is one of genetics, the source of animals, or the source of feed. It could be caused by many things. We are still wondering why this happened, and the issue may require 2 or 3 years and $1 to $2 million to resolve.

NEW TECHNOLOGY

Another area from the regulatory view (although we are not really regulating in this area) is that of microarray technology. In the pharmaceutical arena of this technology, we are beginning to look for better ways to interpret study results—possibly to understand mechanisms for responses and to eliminate the conduct of some studies. Using this technology will be a learning experience.

Currently, I participate in an International Life Sciences Institute group, which collects and attempts to compare across market-ready platforms in an effort to characterize platform responses. The purpose of this effort is to gain a similar experience base and even, in fact, build a standard response library to enable an understanding of what kind of toxic insult might reveal a predictable pattern. It will be years before this database is built.

One of my questions is what animals will be used when specialists validate or characterize this microarray platform response data set. During our last meeting, we discussed whether we should use SD or Wistar rats when trying to compare the response with a single chemical at multiple test sites, to compare platforms. That comparison can be carried farther, and there may be data to address this issue. Differences in the source of animals should be considered, even if you get what can be called a particular strain.

Finally, it is important to determine a course of action for studies in a different strain or source of animal that result in a different signal with an unknown chemical. We need to know with certainty that a different response is due to a

genotypic difference in the animals. We must learn to understand the genetics and what expression changes are associated with what strains or whether to control for this difference in test systems.

GENETICALLY ENGINEERED MICE

We obtain genotypic and phenotypic expression information—response data for response elements, specifically in the p53 knockout and Tg.AC models. For example, we test to learn whether the transgene has been inserted in the Tg.AC model. In this process, we have discovered that even with a genetic marker, it is possible to be fooled. In the case of the Tg.AC model, there was a long delay in the response characterization because the genotype marker being assessed was not responsible for the phenotype. Because people were not tracking the genotype that was associated with the phenotype of interest, confusion resulted and the model was almost lost. The animals expressing the transgene suddenly were no longer responding to carcinogen treatment. That example clearly reflects a need to have very good characterization of the genotype and phenotype and identification of a genotype that is responsible for the phenotype of interest.

We also now have the p53 knockout model expressed on three different background strains, and we have no information as to whether those accessory genes influence the expression rate in the different p53 animals and whether they all respond with a particular signal. Within FDA, we are looking at the *Hras2* mouse model to learn whether it has the same potential liability as the Tg.AC, because it was made with a technique similar to the Tg.AC model. To the best of our knowledge today, it does not appear to carry this potential problem.

SUMMARY

In a regulatory setting, there is very minimal information on the genetics of animals. However, that information can be very important from a regulatory perspective. We understand the importance of genetically engineered animals, but we do not necessarily appreciate that this importance can also apply to our standard toxicology models. Perhaps in the examples cited above, if we had the genetic information from the Japanese source of animals in the dose ranging study and the source for that carcinogenicity study, resulting linkage information may have enabled us to resurrect the validity of that study. Of course, it is necessary to know what genetic markers are important to follow.

I believe there are emerging issues regarding genotype and interpretations of results, particularly in the area of microarray technology that we are all rushing into headlong without resolution. We must be careful about what kind of data we collect. We must consider the meaning of an observed effect that no longer appears as a signature signal—consider the source of animals and any other factors that could have influenced that animal's response.

QUESTION AND ANSWER

DR. FESTING: What is the implication of a case in which genetic markers showed that both the Japanese rats and the US study rats were, in fact, genetically different?

DR. DE GEORGE: The implied result is basically the same as the current situation, which is that the study must be repeated. According to that 2-year assay, the doses were not selected properly for that group of animals, which means that we did not learn about the carcinogenic potential of the drug. If the rodent to human dose margin had been huge or the dose had been close to the appropriate dose, we probably would have been able to accept those data. However, in this case, the doses were clearly too divergent. The result is $1 million wasted and 2 years lost, the latter of which is probably more important.

Genetic Evaluation of Outbred Rats from the Breeder's Perspective

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The origin of outbred laboratory rodents can be traced back to the 1500s. In the case of rats, populations of wild-caught animals were kept and bred to supply the blood sport of "rat baiting." In doing so, small groups of rats from the much larger wild population were selected and bred, thereby providing a ready source for use in biomedical research in the 19th century. In the 1890s, a small number of these rats were brought to the United States from Germany to establish a laboratory-maintained research population. These initial animals were later randomly mated at a number of institutions with occasional infusions of a few animals from the wild and pet populations. During the early part of the 20th century, additional selection for a variety of traits, as well as the lack of a purposeful outbreeding system, likely caused a significant reduction in the individual genetic diversity of these noninbred rat stocks, which has continued until relatively recently.

POPULATION MANAGEMENT

Outbred population management was not seriously considered until the 1960s and even then was not widely applied to rodent production. Both commercially and academically, linking subpopulations and even starting new colonies did not include consideration of sampling error or population divergence. It was considered more important simply to begin breeding in a way that was not purposeful inbreeding with the hope that heterozygosity would be maintained. Unfortunately, the current interest in preserving and perhaps increasing heterozygosity in outbred populations, as well as addressing random genetic drift, is "historically"

hampered by the fixation of large amounts of the rat genome across all outbred stocks, compared with natural populations and perhaps humans.

SUPPLY AND DEMAND

Currently, approximately 75% of all rats and mice produced commercially (at least in the United States) are noninbred. Although inbred strains have the most prominent role in transgenic and knockout animal development, here too outbreds are still used for a number of applications. Worldwide, pharmaceutical and contract research organizations consume more than 70% of all commercially produced laboratory animals including rats. Because this demand will likely continue, we need to manage and genetically monitor outbred animals and, in particular, outbred rats correctly.

With proper management as the target, there are several things to remember. As mentioned by other speakers, random genetic drift occurs in outbred populations. Over time, two populations starting with equal gene frequencies of alleles arbitrarily designated as capital *A* and lower case *a* will undergo random genetic drift. Eventually, one population may develop an increasingly greater proportion of a single allele, *A*, and after many generations that allele may become fixed in that subpopulation while the other subpopulation may continue to segregate until such time as the proportion of one or the other of these alleles increases and also becomes fixed.

One of the goals of this meeting has been to consider how to use genetic monitoring and allele frequencies to assess subpopulations for the purpose of determining relatedness and presumably for management interdiction. At Charles River, as elsewhere, we began evaluating subpopulations of outbreds using biochemical markers. As expected, we have seen that many of them are monomorphic. We surveyed the three populations of Wistar Han rats, as shown in Figures 1 and 2, using biochemical and immunologic markers. These populations were from unrelated commercial breeding facilities and represented different sources and dates of acquisition of breed stock. Although some differences do exist, there is striking similarity between the frequency of biochemical and immunologic phenotypes among all three populations. Existing differences are not consistent between subpopulations.

In trying to judge the similarity of two populations based on the distribution of a single marker, one can easily overlook contradictory information if all of the other makers are not considered. Even if a panel of markers is used, judgments regarding the similarity of populations will be limited by which markers are surveyed. The assumption that some standard panel of markers that can easily fingerprint populations for the purposes of authenticating them, as is done with inbred or F1 hybrid animals, does not consider the possibility that the distribution of phenotypes for any given marker can change over time even when comparing populations that are considered to be closely related.

Marker Type	Gene Designation	Allele	Source Pop. 1 (%)	Pop. 2 (%)	Pop. 3 (%)
Biochemical	ES-2	a	78	80	80
		d	22	20	20
	ES-6	a	100	100	100
		b	0	0	0
	ES-10	a	100	80	100
		b	0	20	0
	Pep-3	a	100	100	100
		b	0	0	0
	Hbb	a	29	30	40
		b	71	70	60

FIGURE 1 Wistar Han rat stocks: Partial genetic analysis of three populations using biochemical markers.

To interpret information gathered from phenotype assessment of outbred populations using biochemical, immunologic, or DNA makers, it is necessary to view this information as a whole and to consider not only similarities but also differences in the marker profiles. This inclusive information can best be obtained through the calculation of certain genetic monitoring statistics such as fixation index, estimate of polymorphism, conformity to Hardy-Weinberg equilibrium, and an estimate of average heterozygosity (Hartle 1988). Such computations yield a single number that considers all markers surveyed without unduly emphasizing a single marker, which may not be representative of the amount of divergence between two subpopulations if all possible markers are surveyed.

Marker Type	Gene Designation	Allele	Source Pop. 1 (%)	Pop. 2 (%)	Pop. 3 (%)
Immunologic	RT-1	b	38	70	70
		I	13	20	0
		U	50	10	30
	RT-2	a	63	90	70
		b	37	10	30
	RT-8	a	0	0	0
		b	100	100	100

FIGURE 2 Wistar Han rat stocks: Partial genetic analysis of three populations using immunologic markers.

VARIABLES AFFECTING COMPARISONS OF SUBPOPULATIONS BY GENETIC MONITORING

Sample size is an important variable in any such analysis of subpopulations using an array of markers. Because outbred populations have considerable individual variation, small sample size may not truly yield a distribution reflective of the subpopulation, hence, 10 rats sampled may suggest one distribution whereas 100 rats may provide a different distribution and a better estimate of the subpopulation in question. It is also important to consider the "effective population number" and to make the appropriate corrections when analyzing data (Hartle 1988). The effective population number can be altered by age-related differences in reproductive rates as well as unequal numbers of males and females in a particular breeding scheme. It can also be affected by inequality of litter size due to production practices such as consolidation at birth or limitations placed on litters used for selecting future breeders. Unequal population numbers can also be an important variable inasmuch as population size can change generation to generation depending on production goals and other issues that may increase or decrease the population. Overlapping generations can also affect these analyses because the entire population does not progress to the next generation at exactly the same time.

CAUSES AND AMELIORATION OF GENETIC DIVERGENCE

There are three methods by which genetic divergence occurs. The first method is mutation, which can result from a number of physical/chemical processes. The chance of retention of any mutation in a population is relatively low. The second method is natural selection, which probably has a limited role in laboratory populations especially when rearing practices and the environment are relatively constant. The third method is unconscious selection wherein future breed selection is biased unconsciously by practices such as preferentially breeding good-tempered animals, animals with large litter size, or animals with lack of runted offspring. Such selection practices unconsciously favor phenotypes (and hence genotypes) that contribute disproportionately to the pool of future breeders.

The principal method for minimizing genetic divergence that occurs between geographically separated colonies (that is, subpopulations) is the trading of breed stock (migration) between colonies. Without migration, random genetic drift can be expected to cause at least moderate genetic divergence over time among outbred colonies derived from the same source. Migration of animals between colonies can be viewed as a form of genetic glue that holds colonies together and sets a limit on the amount of genetic divergence that occurs (Hartle 1988). Migration is not without its difficulties inasmuch as other factors such as the potential for microbiologic contamination of existing colonies must be considered in

the migration process. This risk can be minimized by indirect migration to and from a cesarean-derived isolator-maintained foundation colony.

Besides genetic divergence, colonies of outbred animals can differ significantly based on how and how many breeders were selected for the startup of the colony. This degree of difference is sometimes referred to as a genetic "bottleneck" or the founder effect. As can be seen in Figure 3, a comparison of the process of inbreeding with random mating of a colony beginning with 5 or 80 pairs of breeders, the amount of inadvertent inbreeding and hence fixation of alleles overtime can be influenced by the number of breeders selected to start the colony. If too few breeders are selected from the founder colony, the true allele frequencies in that colony will likely be misrepresented through sampling error in the newly established colony. This error can be magnified if a purposeful outbreeding system is not in place to adequately avoid inadvertent inbreeding and thus maintain the diversity of the population (White and Lee 1998). Random mating will not achieve this diversity, particularly if there is only a small number of breeders to choose from.

Another common error that produces nonrepresentative sampling is using entire litters as breed stock when forming a new colony. In addition, if a foundation colony that is linked to subpopulations by a regular migration process of breeders is not present, selection of a single production colony as a source of breed stock for a new colony may also misrepresent the genetic diversity present in all of the production colonies being maintained by an institution or breeder

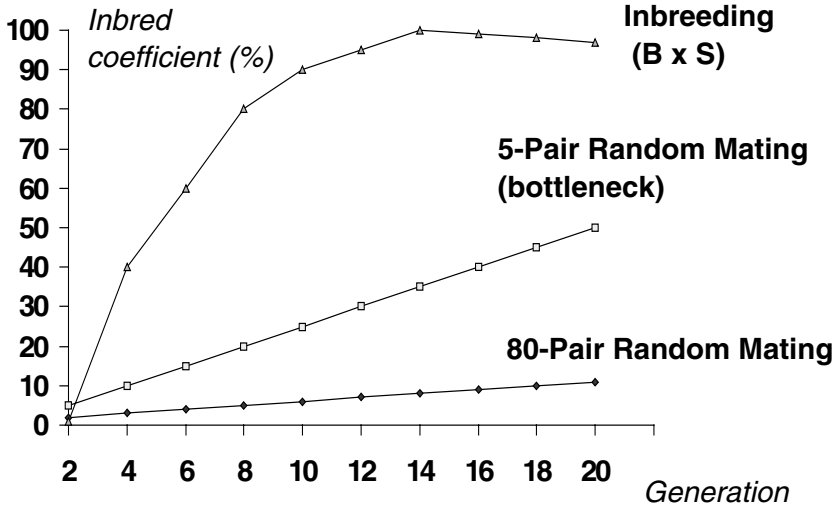


FIGURE 3 Coefficient of inbreeding with different colony size and mating systems.

(Figure 4). In the absence of a foundation colony, all of the production colonies must be sampled to develop a new production colony (Figure 5).

DEVELOPMENT OF A FOUNDATION COLONY-BASED OUTBRED PRODUCTION SYSTEM

The majority of commercially produced rodents are raised in barrier rooms that use very large breeding populations. The average rodent production room at our company is 2200 net square feet of floor area and will house approximately 60,000 rats that produce, in the case of outbreds, about 4000 rats for sale per week. In constructing our foundation colony-orientated outbred production system for CD rats, we began by identifying the 27 existing colonies of these animals worldwide and examining their stocking and production histories. To preserve the maximum amount of genetic diversity, we searched for colonies that had been separated for at least 5 to 10 years and found eight colonies that had been separated from each other (no infusion of new breed stock) for 12 to 22 years. We then selected 100 individuals (50 males and 50 females) from these eight colonies and placed them in a barrier room. Each group of 100 animals was designated as a separate line, and we began a circular-paired mating system from

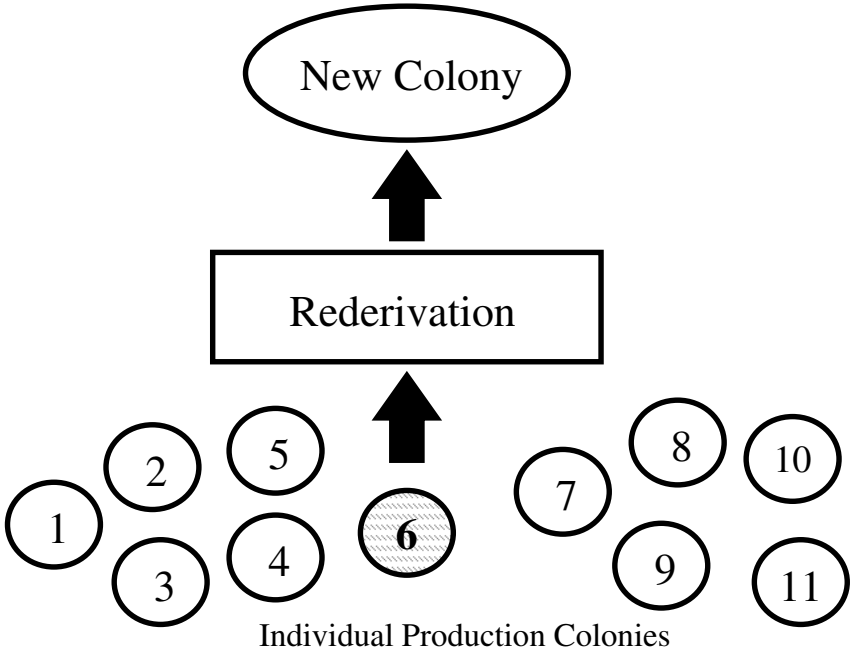


FIGURE 4 New colony set-up using only one of 11 colonies.

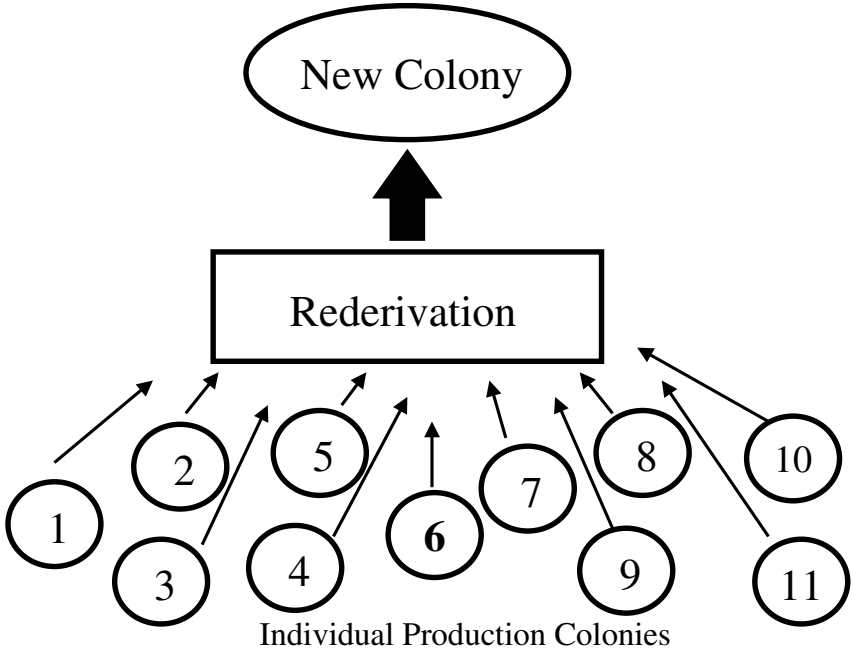


FIGURE 5 New colony set-up using animals from all colonies.

which we derived pregnant females for rederivation and subsequent selection of breed stock for population of new barrier rooms. We selected one pup per litter from 200 litters so as not to overrepresent any breeding pair (Figure 6). We repeated this process for each new breeding colony that we set up and began the process of closing breeding colonies and resetting them up with new stock using this system. We chose cesarean rederivation over embryo transfer rederivation because of the ease by which the process can be conducted in rats, which was important given the large number of pregnant females that had to be sampled.

The overall process to stock a barrier production room using a barrier room-maintained foundation colony was as follows. Pups were obtained by hysterotomy under aseptic conditions using a dual laminar flow hood technique. Rederived pups were aseptically transferred into 3-foot semirigid isolators and cross-fostered onto lactating females of defined flora status (Charles River Altered Schaedler Flora [CRASF]). Extensive health monitoring was conducted on both the environment and the foster mothers within the isolators throughout the course of the postpartum and weaning periods. Eight- to 12-week-old pups were packed into self-contained transport isolation shipping devices using aseptic technique and flown to the production site. Upon receipt at the production site, the integrity of the isolators was examined, and the shipping isolators were then

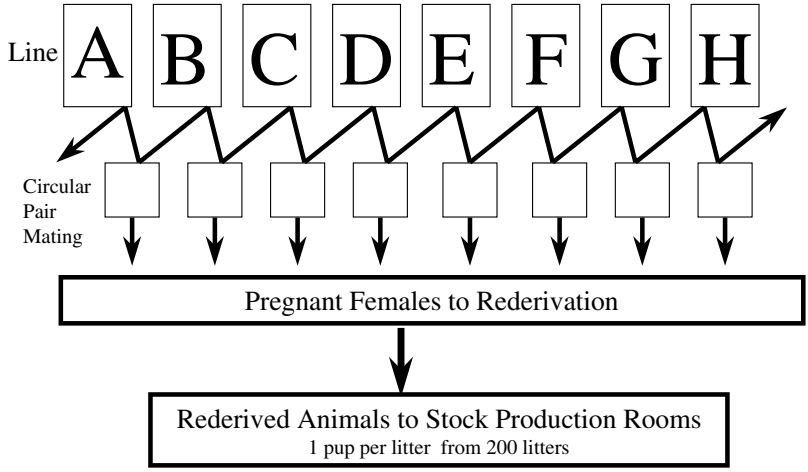


FIGURE 6 Crl:CD®(SD)IGS BR foundation colony system for producing stock for forward migration.

connected to a transfer isolator for unpacking. The animals were then transferred into the production room using a transfer isolation port built into the room that was connected to the transfer isolator using a plastic sleeve. All steps of the process were done using aseptic technique so that animals received into the barrier room retained their CRASF status. Transferred animals were used to start up the new production colony.

From a microbiologic standpoint, the maintenance of foundation colonies in a barrier production room that is entered by people poses a potential microbiologic risk especially if a regular forward migration process is conducted in addition to colony startups. To eliminate this risk, it was decided to rederive a colony of 250 breeding pairs representing the eight lines into 20 semirigid isolators. Each isolator contained up to 13 cages of breeding pairs, 12 cages holding future breed and stock, and two cages holding animals used for health monitoring of the isolator. In the case of the CD IGS foundation colony, the 20 isolators produce weanlings each week that are used for future breed replacement, forward migration, colony setup, and limited sale of overproduction for specialized customer use (Figure 7). In addition to the CD rat outbred foundation colony, there is a foundation colony for Wistar Han rats and CD-1 mice as well as foundation colonies for all of the inbred strains produced by the company. More than 200 isolators of varying sizes are used for foundation colony maintenance with another 1200 isolators being used for animal production, special animal services, and rederivation.

The 20 CD rat foundation colony isolators are maintained by a circular-paired mating system within each isolator and a migration system whereby fe-

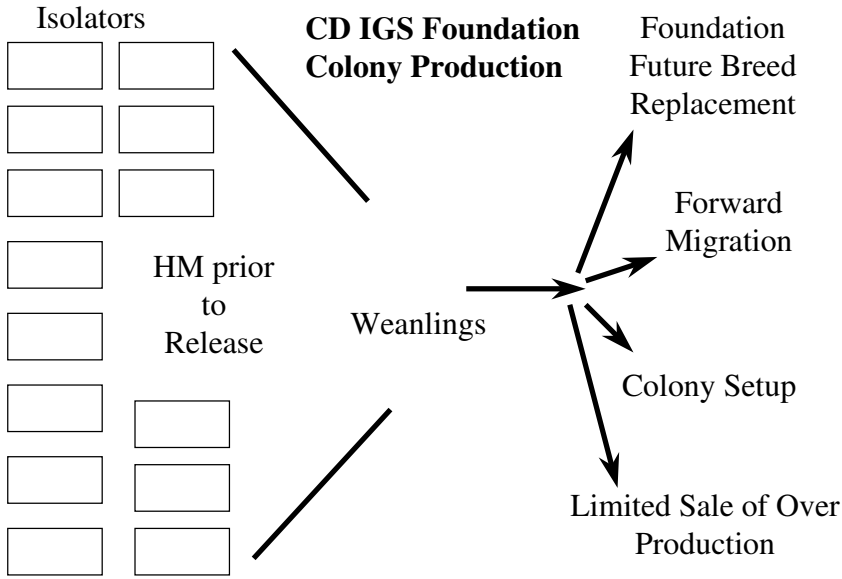
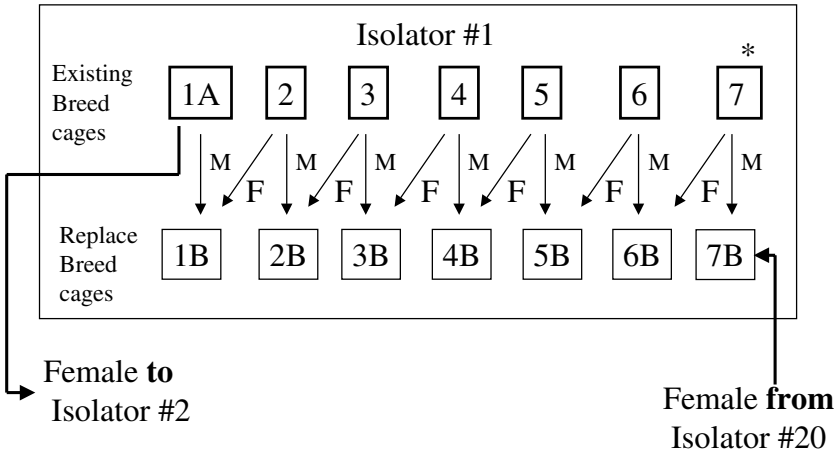


FIGURE 7 Schematic of foundation colony and its uses.

males from one isolator are migrated to another at designated times during the calendar year (Figure 8). These isolator transfers are linked to health monitoring, allowing isolators receiving transferred animals to be placed on quarantine hold until the results of health monitoring of that isolator have been obtained (Figure 9). This monitoring provides additional assurance that any change in the microbiologic status of the animals within an isolator can be detected before transfer of animals to new or existing colonies.

Forward migration of breed stock occurs from the foundation colony to the production colonies at 3-year intervals with 25% of the production male breeders being replaced in each colony by this migration process. Migrations are staggered over a 3-year period so that each year, one third of the colonies receive migrated animals (Figure 10). This process links all of the colonies to the foundation colony proactively making corrections without waiting for genetic monitoring to detect drift and a course of action to be undertaken.

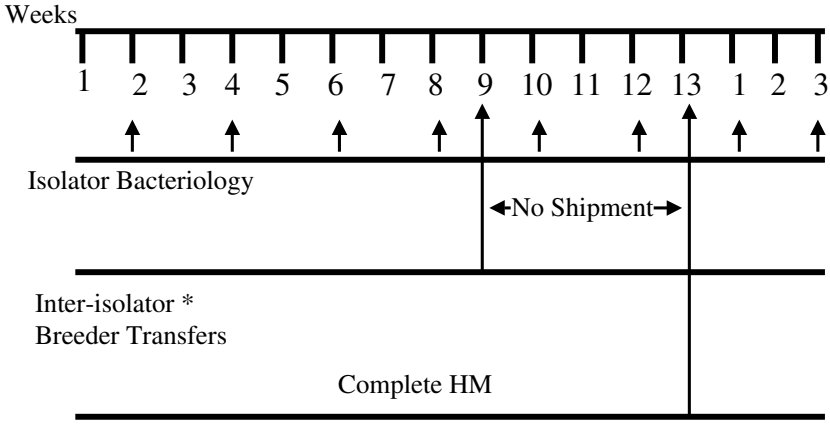
Unlike some migration systems, the genetic management of outbred foundation colonies by our company also includes backward migration (in-migration) at 5-year intervals. Each year, 1% of the foundation colony breeders is replaced by breeders brought back from the global network of production colonies of that stock. Animals received are mated, and the pregnant females undergo rederivation as previously described. Progeny are then held in extended quarantine during which time comprehensive health monitoring is conducted to ensure that



*Example only - each isolator has at least 12 breed cages

FIGURE 8 IGS outbred rat foundation colonies breed pair replacement.

the appropriate microbiologic state is maintained. Replacement breeders are then introduced into the foundation colony (Figure 11). This process of in-migration helps maintain genetic diversity in the foundation colony and ensures that the foundation colony reflects the variation found in the production colonies.



*Frequency of one fourth of the isolators per 13 weeks

FIGURE 9 IGS foundation colony management environmental and health screening.

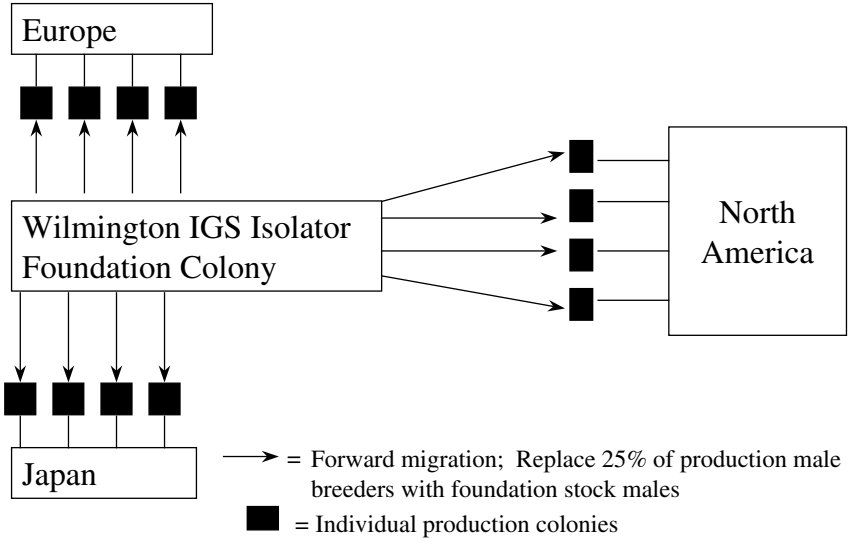


FIGURE 10 IGS: Forward migration—every 3 years.

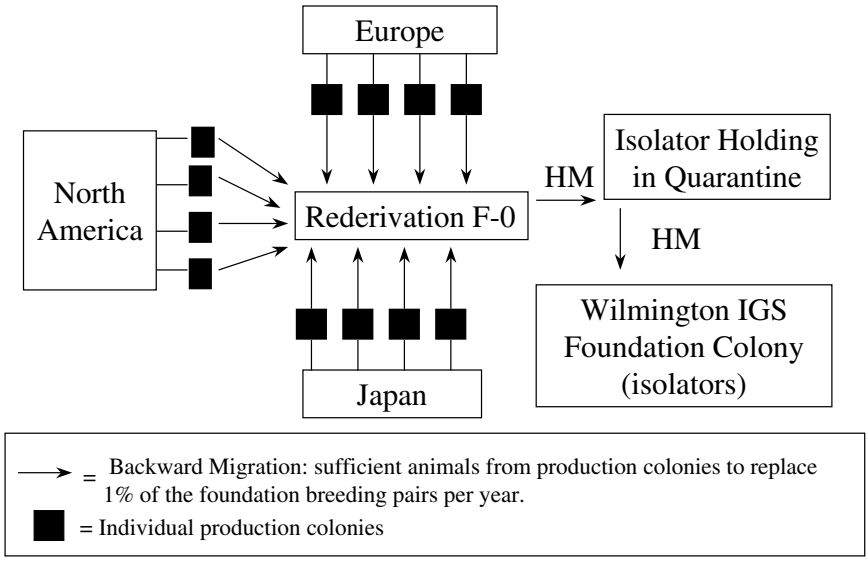


FIGURE 11 IGS: Backward migration—every 3 years.

Within the individual barrier production rooms, we utilize a line-breeding system and rotate female breeders between lines. We have developed a number of operational procedures to minimize inadvertent inbreeding while ensuring consistency of production. Animals released for sale from production colonies are a mixture of all of the lines and hence a representative sampling of the entire colony. We refer to the overall genetic management system that uses an oubred foundation colony with forward and backward migration as the IGS system (White and Lee 1998).

CONCLUSION

I urge this group, as they consider methods for monitoring outbreds, to remember that "outbreds aren't inbreds." We are not looking for authenticity when monitoring populations but for similarity. We are trying to preserve the great degree of individual variation in the population and hence should not expect individual uniformity as with inbred animals. Monitoring and management of outbred populations inevitably comes down to the issue of comparisons. It is unlikely that there is a single marker or set of markers, or a specific value in a population genetic statistic calculated from the frequencies of such markers, that can be used as an absolute cutoff in determining similarity of subpopulations. I urge the group that before undertaking such a determination, they include population geneticists in the deliberations.

So far, no one has discussed the role of cryopreservation in managing outbred populations. Some would argue that simply cryopreserving the foundation colony and using that for restarting or migration purposes would be the ultimate solution. Cryopreservation is important for preventing disastrous loss. One can argue, however, that cryopreservation is a selection method because only those embryos that can survive cryopreservation will survive into the reconstituted generation. It is unclear what traits, if any, might be linked to cryopreservation. If one is to maintain the diversity of an outbred population, some form of foundation colony of live animals is likely required even though cryopreserved animals from such a colony may be maintained as an insurance policy or be included in a backward migration program to provide some temporal refocusing of the foundation colony.

Overall, outbred rats will continue to play a role in research for the foreseeable future. The preservation of heterozygosity in outbred stocks and the linking of subpopulations are critical to the production of outbreds. Although genetic monitoring of outbreds can be used to compare subpopulations, such comparisons are relatively qualitative, are time sensitive, and cannot be depended on to completely quantify all genetic drift that has occurred. Proactive genetic management is the only practical way to ensure similarity of subpopulations.

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QUESTIONS AND ANSWERS

DR. KAGIYAMA: Please restate why we need migration between subpopulations of outbred stocks.

DR. WHITE: If you maintain subpopulations without migration, you will experience genetic divergence. If you have an outbred stock such as Sprague Dawley or Wistar Han and you have 20 colonies (subpopulations), they are all going to drift independently unless you migrate animals between them to make them one functional colony. One could argue that you need only outward migration, but that argument is based on the assumption that whatever you call your reference colony will not vary significantly over time and will reflect all of the other subpopulations. We believe that some inward migration or back-migration is necessary to completely link the foundation with all of the production colonies (subpopulations), which are providing animals continually to the biomedical research community. It is the only practical thing you can do to proactively counteract genetic drift. By the time you obtain genetic monitoring data, the damage will have already been done. The practical approach is to be proactive in colony management and use genetic monitoring as a qualitative assessment of success.

DR. NOMURA: Please explain the idea of international genetic strains. After you mixed animals from different colonies to form the foundation colony, did you monitor them genetically? I would also like to see the actual genetic frequency data of the system you have theoretically described.

DR. WHITE: In developing the foundation colony, we made a decision to retain as much potential genetic diversity as existed in the production colonies at the time the foundation colony was formed. We looked for colonies that had been separated the longest without infusions of new breed stock, restarts of colonies, or the addition of animals into the breeding population by any other means. As you know, in commercial production, sometimes a colony will be developed and be in production for awhile and then be phased out because there is not sufficient demand for the animals being produced. Of the colonies available at that time, we were looking for ones that had been separated the longest, since they would have been more likely to have mutations or alleles that had become fixed, and thus, have made them different from other subpopulations of the same stock. In concept, this process is similar to construction of an F1 hybrid through a multiple F1 hybrid cross. We, in fact, did survey the individual colonies selected using

biochemical and immunologic markers; however, I did not bring those data with me today.

DR. SHEK: With respect to the markers for monitoring, we have biochemical and immunologic markers and are now looking at DNA fingerprinting and microsatellites for monitoring the IGS colonies. However, we and our consultants are not convinced that we currently have a set of markers that are extensive enough to do meaningful monitoring. We are continuing to look at minisatellites and trying to develop a more extensive set of microsatellites for comparing genetic divergence between colonies. If we use markers that are not highly polymorphic (for example, if we try to use microsatellites where there is a lot of band sharing), we will always get the results we want, which is that there is no divergence among our subpopulations because there is so much polymorphism there initially. This is an important point to consider even if biochemical markers are used. We are in the process of developing more effective DNA monitoring and results analysis techniques that should aid in comparison of genetic divergence between colonies.

DR. JACOB: One of the key points is how much monitoring is done. In my laboratory, if we are going to use a strain for something, we use a minimum of 600 markers. Hundreds are required for detecting diversity. At some point, you must characterize the amount of diversity to know how much diversity is there and to be able to construct the diversity in a population that you are seeking. You lose alleles every day you delay.

DR. SHEK: I disagree. Population geneticists have looked at this problem mathematically, and we are following their recommendations. Managing genetic divergence is definitely more important then the method or systems for monitoring it since monitoring only detects what has occurred and does nothing to counteract genetic divergence. Moreover, we are not trying to increase or decrease genetic diversity by artificial manipulation, but simply trying to preserve it.

Concept for Establishment of Rat Outbred Global Standard Strains

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INTRODUCTION

Accuracy and quality standards for reagents and measuring scales are very important for the reproducibility of experimental results, and methods to verify such standards must be established. The need for verification also applies to experiments involving the genetic quality of laboratory animals.

Outbred rats are the most common animals used in drug safety testing at this time. Reproducibility of animal experimental results obtained using outbred rats can be expected only if the genetic quality of the rats is guaranteed. However, a genetic testing system to verify the genetic quality of outbred rats has not been established.

Outbred rats are often used in bioassays such as carcinogenicity studies. According to the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (DeGeorge 1997), future carcinogenicity testing applications must consist of one set of animal studies for joint new drug applications, which must be submitted simultaneously in each country. The animals for this purpose must be "carefully selected standardized outbred rats of the same strain," which can be used on an international level in safety studies to achieve reproducibility and comparability of results. This condition requires reliable genetic and microbiologic quality standards and test methods. In particular, a genetic testing system that can monitor the quality of these outbred rats (technically difficult to achieve in the past) must be established.

HISTORY OF GENETIC QUALITY CONTROL

As shown in Figure 1, from the time the laboratory animal modernization movement started in 1950 until 1980, there were no quality concepts and test methods to confirm genetic profiles. Animals were used only on the basis of strain names. At the end of the 1970s, the genetic profile concept and monitoring system as a genetic testing system using biochemical and immunologic markers for inbred strains was established, and mice and rats covered by the genetic monitoring system became available. In 1979, the ICLAS Monitoring Center was founded in CIEA (Figure 1).

In 1985, the genetic profiles of six strains of Wistar inbred rats from various countries around the world were examined, and different results were obtained (Figure 2). Before this study, they were considered to be the same. These results revealed to most users the importance of genetic quality testing. However, outbred rats used at present are not subject to genetic quality control because such control is difficult compared with inbred or hybrid rats. The genetic background of outbred stocks is not clear, and genetic changes leading to genetic drift might occur due to an accidental infection.

QUALITY STANDARDS

The quality standards or specifications for these animals must be set by users of the animals (that is, pharmaceutical companies and the regulatory authorities) and not by breeders. In addition, the regulatory authorities and pharmaceutical companies must use the same scales to evaluate animal experiments. Our objective is to develop a testing system by which the genetic background of the outbred rats can be clearly understood so that it is possible to perform genetic control of the outbred stocks and to produce the animals in large numbers with a uniform genetic structure and permanent characteristics. Our objective is to establish the generational stability of characteristics.

Global standards of outbred rat strains cannot be established without evaluation of genetic profiles as a scale; however, no methods have been available to establish such profiles in outbred rats. In 1990, we studied outbred stocks of rats using the genetic monitoring system, and genetic testing methods to confirm the genetic quality of outbred stocks were established (Katoh and others 1998). The results of tests on three Wistar (A, B, and C) and 2 SD (D and E) strains showed different genetic profiles in animals with the same strain name, which was considered to be due to the bottleneck effect or artificial selection, as seen in Figure 3, A and B.

CONCEPT OF GLOBAL STANDARDS FOR OUTBRED RATS

As shown in Figure 4, CIEA concluded in 1999 that it is essential to establish global standards of outbred rats based on the following concept. In using the

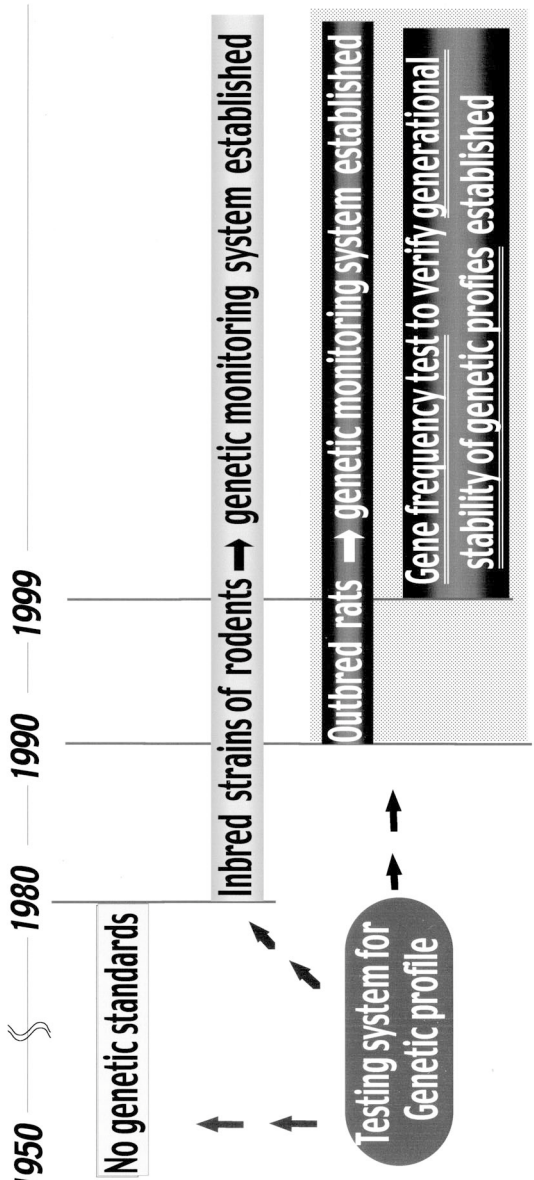
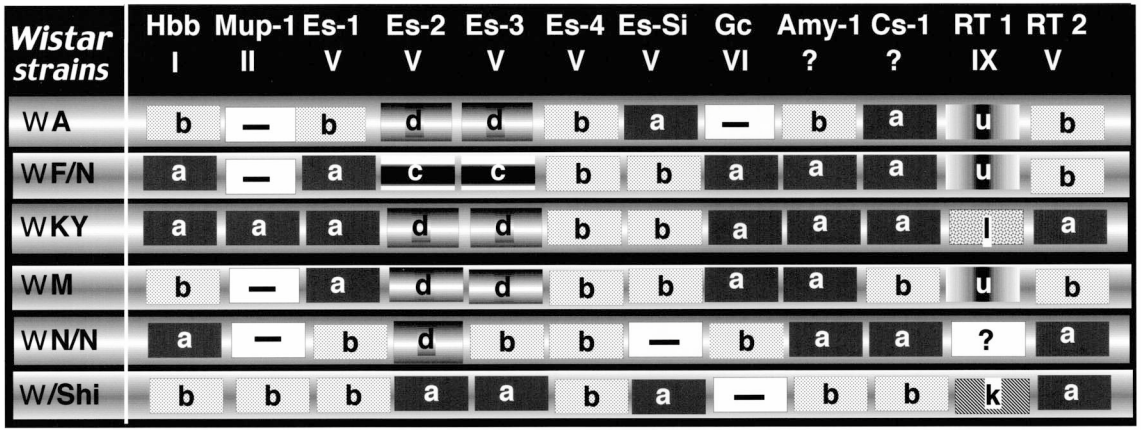


FIGURE 1 Historical background of genetic quality of laboratory animals (rodents).



Wistar strains	Hbb I	Mup-1 II	Es-1 V	Es-2 V	Es-3 V	Es-4 V	Es-Si V	Gc VI	Amy-1 ?	Cs-1 ?	RT 1 IX	RT 2 V
WA	b	—	b	d	d	b	a	—	b	a	u	b
WF/N	a	—	a	c	c	b	b	a	a	a	u	b
WKY	a	a	a	d	d	b	b	a	a	a	l	a
WM	b	—	a	d	d	b	b	a	a	b	u	b
WN/N	a	—	b	d	b	b	—	b	a	a	?	a
W/Shi	b	b	b	a	a	b	a	—	b	b	k	a

Wistar strains	Origin			Generation
WA	Laboratory Animal Centre,MRC,	UK	1980	F?+ 9
WF/N	National Institutes of Health,	USA	1982	F33+ 6
WKY	Takeda Chemical. Industries,	Japan	1984	F37
WM	National Institute of Genetics,	Japan	1979	F80+14
WN/N	National Institutes of Health,	USA	1982	F93+ 7
W/Shi	Shionogi & Co.,	Japan	1984ß	F64+ 5

FIGURE 2 Locus and linkage group of Wistar (W) strains (1985).

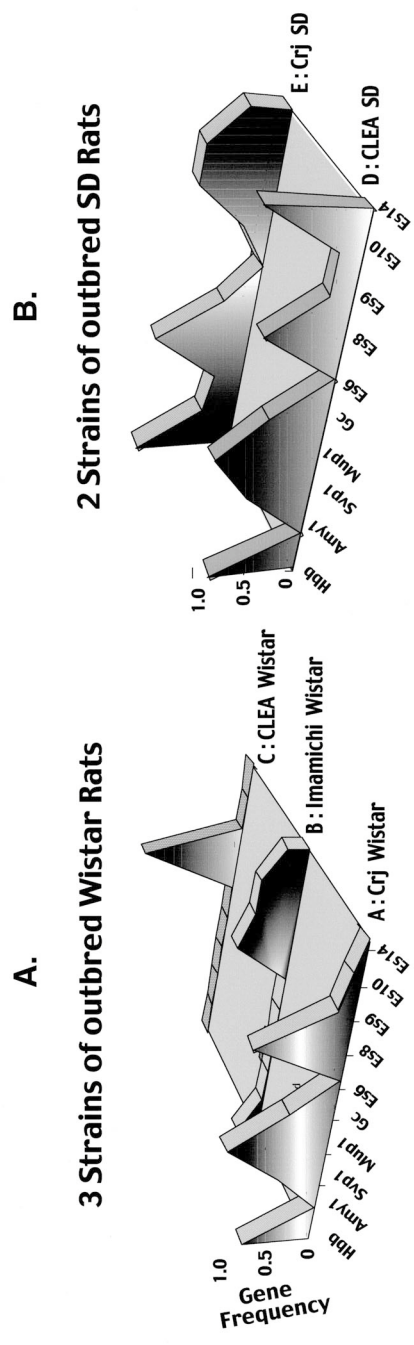


FIGURE 3 Comparison of gene frequencies (1990).

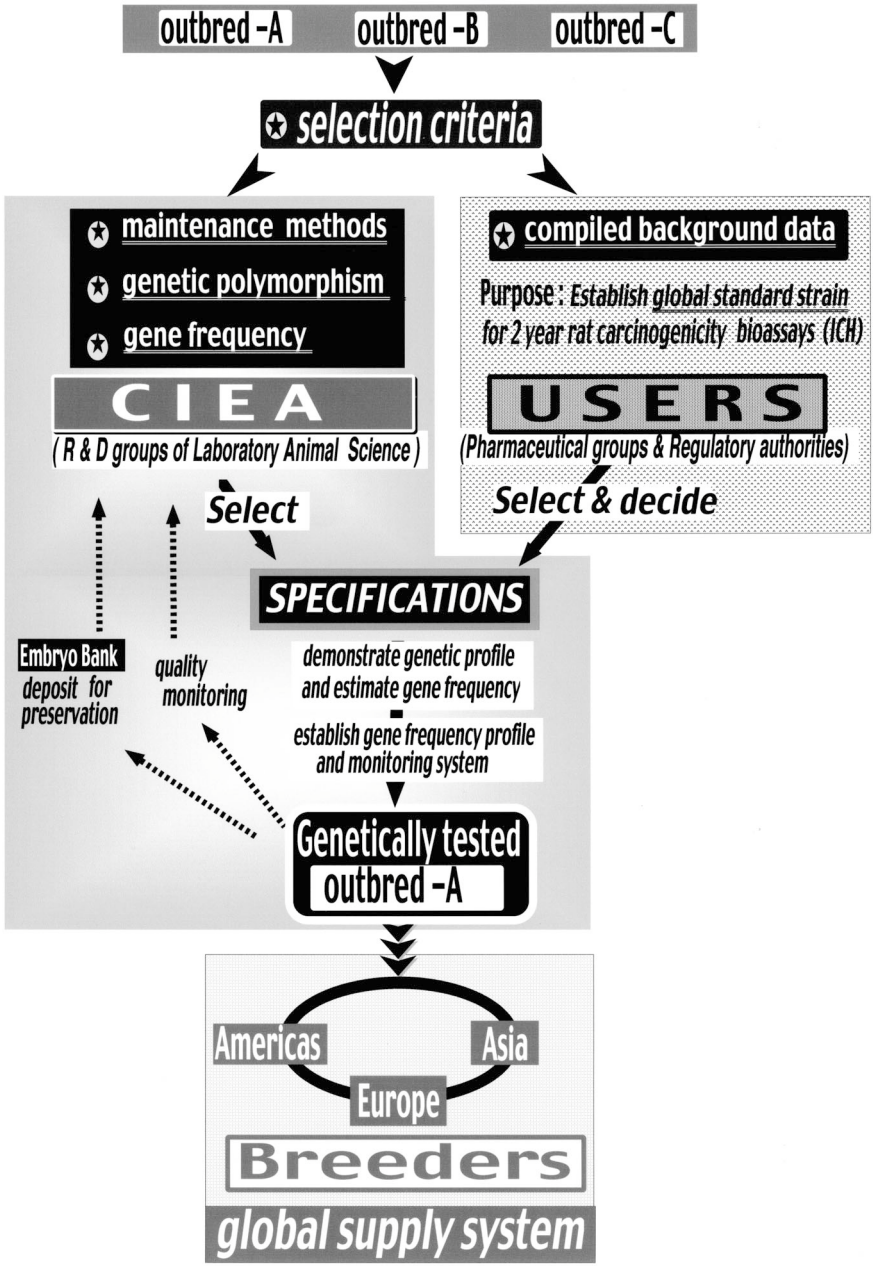


FIGURE 4 Concept for establishing global standards of outbred rats.

several theoretical methods available (Katoh 2000) for global standardization of outbred stocks (such as the multicross hybrid method), the great amount of time required to achieve results is not practical. Not only one but several global standard strains are required. It is necessary to prepare standard strains as soon as possible by selecting the most suitable strains of outbred rats from the several strains available at present. The selection criteria are established by the users and laboratory animal research and development (R&D) group as follows:

- Specifications (standards) Selected by Users Based on the Objective
  - ✶ Compiled background data are used as selection criteria showing that the animals are appropriate for the objective (2-year carcinogenicity study on rats, in this case).
- Selection Criteria for the Laboratory Animal R&D Group
  - ✶ Reliable genetic control must be possible by some method (rotation system) for maintenance of the outbred stock (Figure 5).
    - ▮ Establish a production and maintenance system of colonies to maintain the quality standards (genetic profile).
    - ▮ Establish reliable genetic test methods for evaluating whether the production and maintenance system of the colonies is appropriate.
  - ✶ Genetic polymorphisms
  - ✶ Generational stability of gene frequency
    - ▮ Stability of the gene frequency profile must be assured over time by the established test methods.

SELECTION OF A GLOBAL STANDARD OUTBRED RAT STRAIN

One standard strain was selected by the following method based on the concept described above. As shown in Figure 6, Wistar Hannover stock maintained in RCC by the rotation system was selected on the basis of genetic characteristics and the opinion of users. Four colonies from RCC, CLEA, M&B, and Taconic underwent the gene frequency test in 1993, 1998, and 1999. As can be seen in Figure 7, the gene frequencies shown by various markers were stable in the stocks.

SUMMARY

Genetic quality of outbred rats can now be standardized, which was impossible in the past. CIEA has established the world's first method of assessing the genetic quality of outbred rats using the gene frequency test (including the embryo bank system) to verify generational stability of genetic profiles of outbred rats. With the development of this genetic testing and monitoring method, it has become possible to establish a genetic control system (rotation system shown in Figure 6) for outbred rats and to confirm reliably that Han lbm:WIST rats are

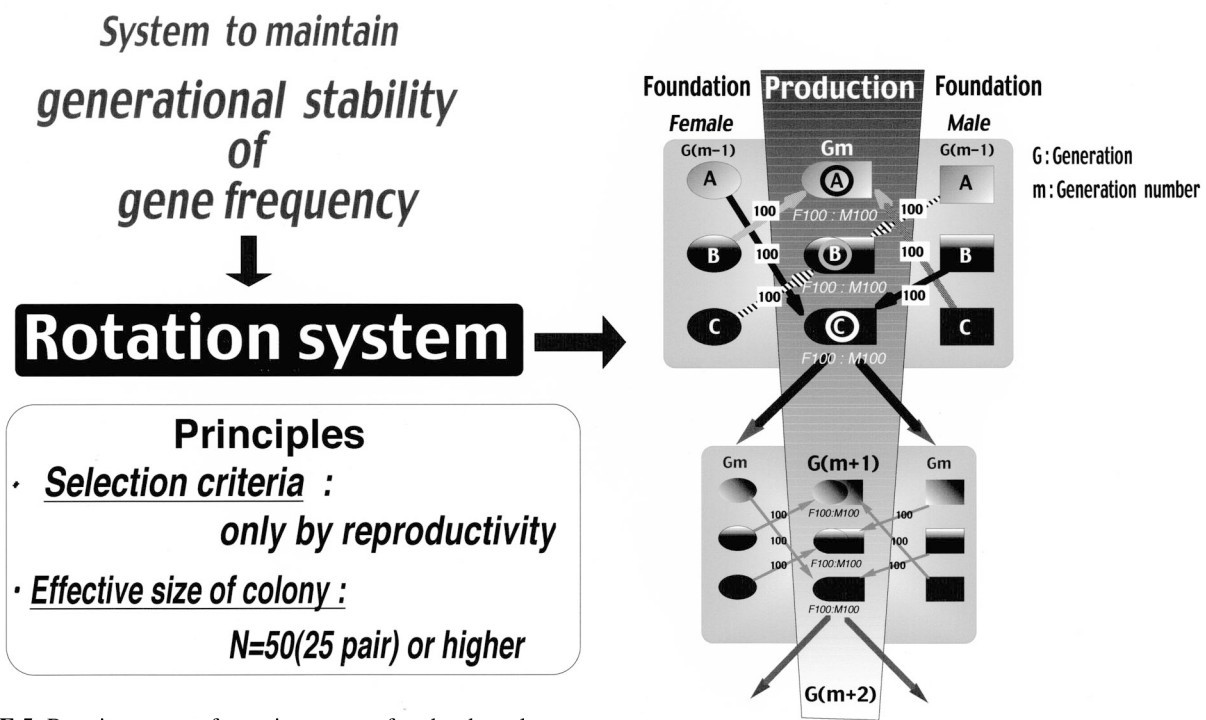


FIGURE 5 Rotation system for maintenance of outbred stocks.

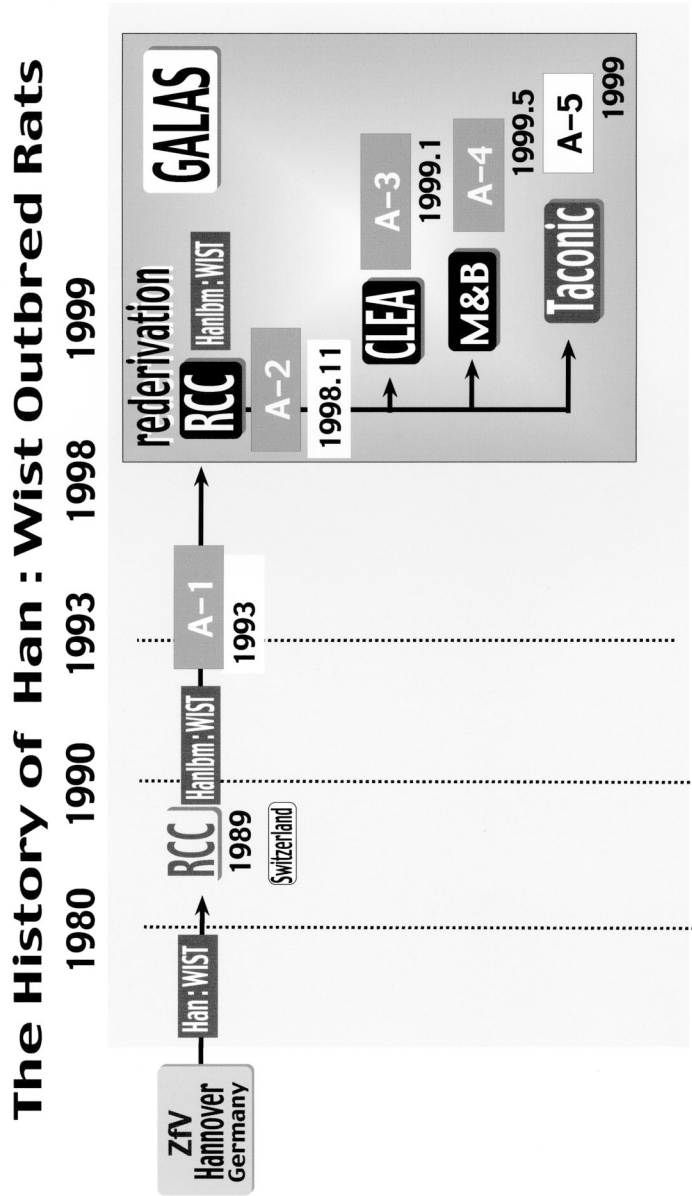


FIGURE 6 The history of Han:Wist outbred rats.

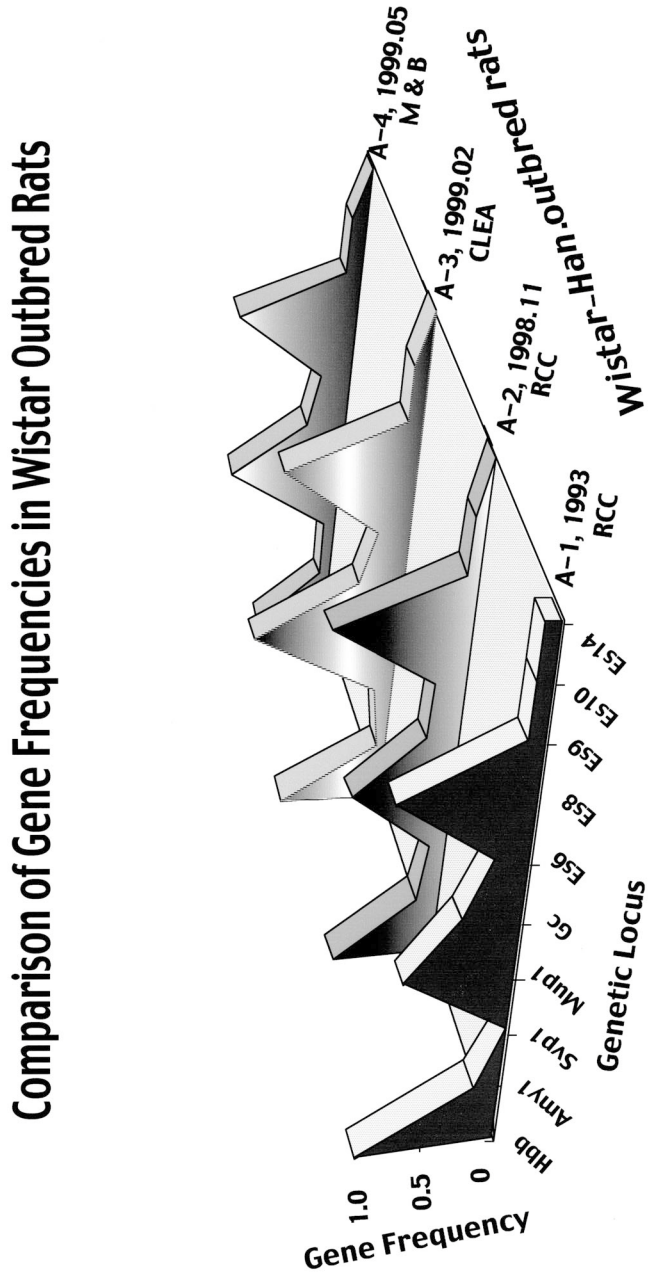


FIGURE 7 Comparison of gene frequencies in Wistar outbred rats.

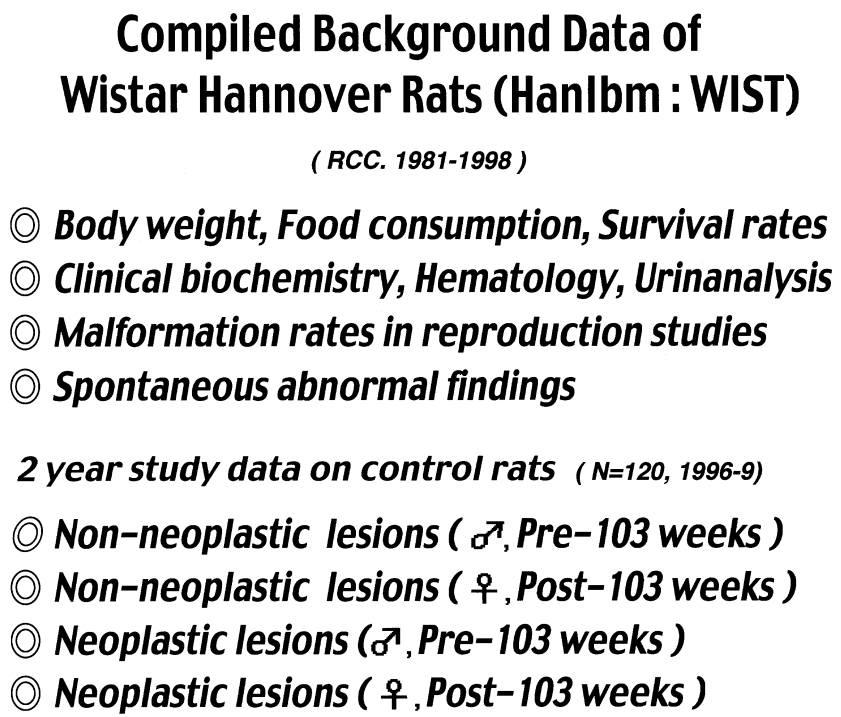


FIGURE 8 Compiled background data of Wistar Hannover rats (Hanlbm:WIST)

appropriate as a standard strain. More than 1,000 pages of data on spontaneous abnormal findings, malformation rates, and 2-year carcinogenicity studies (including control data) as required for ordinary toxicity tests have been collected on Han ibm:WIST outbred rats since 1981. These background data have served as selection criteria for users, as shown in Figure 8. The selection criteria for genetic quality standards (Figure 4) have also been met. It should be possible to confirm from discussions with the users that the Han ibm:WIST outbred rat can be used as one of the standard strains of rats for evaluation of carcinogenicity based on a general evaluation of the data.

We plan to establish global standard rats for the 2-year carcinogenicity bioassay on rats specified by ICH as well as an integrated system for global distribution of these rats as follows:

- Establish genetic control of outbred rats.
  1. Maintain and produce the animals, applying the rotation system on the basis of genetic quality control.

2. Confirm genetic profiles and generational stability by a genetic monitoring system.
3. Preserve the foundation stocks by cryopreservation for emergencies.
4. Establish a global distribution system of standard outbred rats.

A global distribution system of quality controlled standardized outbred rats covering Europe, the Americas, and Asia must be established. Breeders that can assure reliable quality control must be selected in each region, and an alliance should be formed to supply outbred rats of uniform quality.

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Necessity of Genetic and Microbiologic Quality Network from the Pharmaceutical Industry's Perspective

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BACKGROUND

Novartis Pharma, a global pharmaceutical company, has selected the Wistar Hannover rat as the standard stock for toxicology studies. The Laboratory Animal Services Group of the company expects global vendors to ensure the uniformity of animal quality between breeding sites. The guaranteed interchangeability of data obtained at each Novartis site also depends on all sites using rats supplied by the same vendor (Table 1).

ISSUES RELATED TO THE QUALITY OF OUTBRED MICE AND RATS

Genetics: Genetic Drifting and Bottleneck in Outbred Stocks

The genetic profile of a Wistar Hannover rat stock examined in 1993 is shown in Table 2. Because the HanIbm:WIST rat revealed a typical outbred gene frequency, we decided to use the rat for toxicology studies. We would now like to know how we can guarantee that the genetic profile has not changed and will not change in the future.

In Figure 1, an embarrassing experience of genetic drifting is described (Katoh and others 1991). The investigators compared the gene frequency of 23 alleles in the three Wistar stocks, A, B, and C, supplied by three different breeders. This figure summarizes the results on eight esterase-related alleles. Surprisingly, the gene frequency was quite different among the three Wistar rats, and no one would have recognized such genetic drifting if no monitoring had been done.

TABLE 1 Background

- Novartis has selected the Wistar Hannover outbred rat as the standard stock for toxicology studies.
- Novartis expects global vendors to ensure uniformity of animal quality between breeding sites.
- Interchangeability of data between Novartis sites is anticipated as long as all sites are using rats supplied by the same vendor.

- "Quality network" between breeding sites has been established but depending on the vendor.
- Interchangeability of data is not always guaranteed between Novartis sites.

An example of bottleneck in maintaining an outbred mouse stock is shown in Figure 2 (Saitoh and Esaki 1985). The breeder producing ICR mice was faced with an infectious disease outbreak. To rederive the colony, cesarean section was performed. It is speculated that either the number of dams involved or the selection of breeding lines may have been incorrect, and the gene frequency of the hemoglobin beta chain reversed in the renewed colony. Customers complained to the breeder of significant changes in the sensitivity to chemicals and the baseline data on malformation. It is likely that more serious changes related to genetic characteristics are hidden.

Aside from inappropriate breeding and genetic contamination, evolution caused by mutation is unavoidable in any animal population. Therefore, it should

TABLE 2 Genetic Profile of HanIbm:WIST (Excerpt)

Animal	Chromosome and *Allele*									
No. (male)	1 Hbb	2 Amy 1	3 Svp 1	5 Mup 1	8 Es6	14 Gc	19 Es1	19 Es2	19 RT2	20 RT1.A
1	a	a	a	b	a	a	b	a	a	lu
2	b	a	a	b	ab	a	b	a	a	au
3	b	b	a	ab	ab	a	a	a	a	lu
4	b	a	a	ab	a	a	a	a	a	u
5	a	a	a	b	b	ab	b	a	a	al
6	b	a	a	ab	a	ab	a	ad	a	al
7	b	a	a	b	ab	a	a	a	a	al
8	b	a	a	a	a	a	b	a	a	lu
9	b	a	a	ab	ab	ab	a	ab	a	au
10	b	a	a	ab	b	a	a	ad	a	u

NOTE: Checked by ICLAS Monitoring Center-Asia on August 5, 1993.

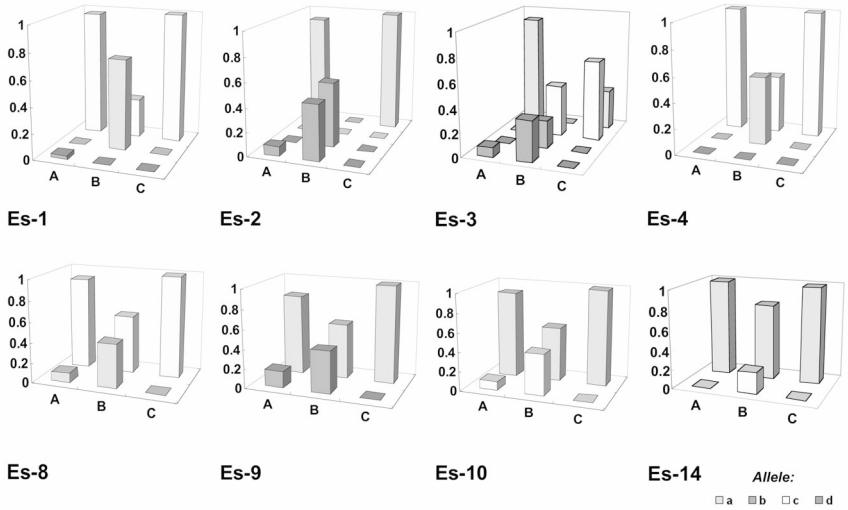

FIGURE 1 Gene frequencies in three Wistar rats, stocks A, B, and C. Reprinted with permission from Katoh H., S. Wakana, S. Utsu, and J. Yamada. 1991. Studies on the genetic monitoring of outbred mice and rats: A survey granted by the Ministry of Education, Science and Culture [in Japanese]. Tokyo, Japan.

be emphasized that monitoring of gene frequency by generation as well as by breeding site is necessary to assess the consistency of outbred stock. However, criteria have not been established for the gene frequency of each outbred stock. We also do not know whether and to what extent the difference in gene frequency is within an acceptable range of diversity. Nevertheless, it is necessary to evaluate the genetic quality of outbred stock not by the stock or vendor name but by actual monitoring results.

Microbiology: Health Profile and Checking Methods

From the viewpoint of global harmonization, three issues have been identified: (1) lack of a common, established health profile; (2) differences in sensitivity and specificity resulting from the variety of checking methods; and (3) lack of reliable monitoring results produced by inappropriate sampling. At this time, I would like to address the profile and checking methods.

For the health profile, we would like breeders to share basic monitoring items, that is, a minimum health profile for periodical monitoring. More items may be requested depending on regional situations such as biosecurity, prevalence of infectious diseases, and regulatory requirements.

For the checking method, we recommend no restriction because the method should be continually improved by experts. Instead, we would encourage refer-

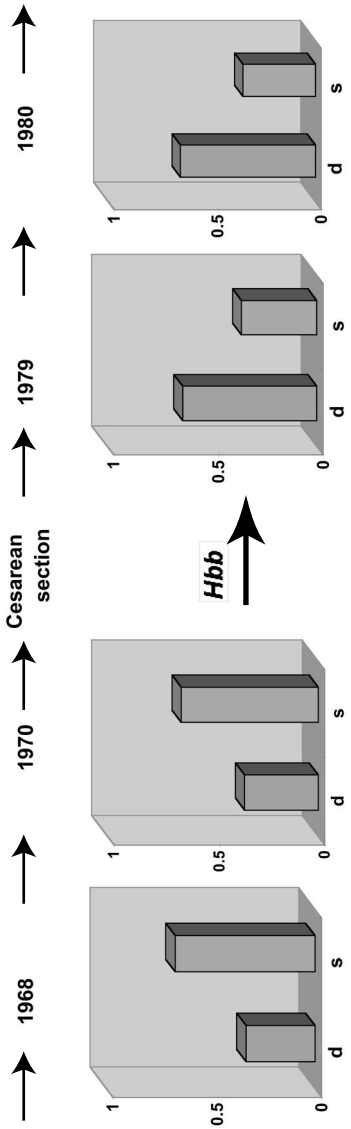

FIGURE 2 Bottleneck occurred in an ICR colony at rederivation. Reprinted with permission from Saitoh, M., and K. Esaki. 1985. Multicross hybrid animals [in Japanese]. Jikken-igaku 3:564–566.

ence organizations to supply reference substances such as validated antigens and antisera. I believe this is the most practical approach to harmonization without jeopardizing scientific freedom.

Concerning the minimum requirement for monitoring, our group studied the selected profiles adopted by the three regional reference organizations in 1996: the FELASA, Microbiological Associates in the United States, and the ICLAS Monitoring Center in Asia. In Table 3, the results on serology for rats are presented. We were not able to prioritize among the three organizations' profiles because each had its own rationale and selected profiles reflecting the needs of the region. However, we scored the profiles as shown in the right column and finally considered 3A as the "minimum requirement" and 2A or 1A plus 2B as "recommended profiles."

We also scored the items for bacteriology, as shown in Table 4. Unfortunately, Microbiological Associates had no checking services for bacteriology and parasitology. We considered 2A as "minimum" and 1A as "recommended."

In Table 5, the result for rats are summarized. The 13 items on the left were taken as "minimum requirements," including Sendai virus, sialodacryoadenitis (SDA) virus, *Mycoplasma pulmonis*, *Bordetella bronchiseptica*, *Corynebacterium kutscheri*, *Pasteurella pneumotropica*, *Streptococcus pneumoniae*, *Salmo-*

TABLE 3 Microbiologic Monitoring Items for Rats (Serology)

Item	FELASA (Europe)	Microbiological Associates (US)	ICLAS-Asia (Japan)	Score
Sendai virus	A	A	A	3A
Sialodacryoadenitis virus	A	A	A	3A
Pneumonia virus of mice	A	A	B	2A
Mouse encephalomyelitis virus	A	B	B	1A+2B
Mouse adenovirus	X	B	A	1A
Minute virus of mice	X	X	B	0A
Kilham rat virus	A	A	B	2A
H-1 virus	A	A	B	2A
LCM virus	X	B	X	0A
Reo 3 virus	A	B	B	1A+2B
Hantavirus	A	B	A	2A
Rat cytomegalovirus	X	B	X	0A
Mycoplasma pulmonis	A	A	A	3A
Mycoplasma arthritidis	X	B	X	0A
Clostridium piliforme (Tyzzer)	A	B	A	2A
CAR bacillus	B	A	B	1A+2B
Toxoplasma gondii	A	X	X	1A
Encephalitozoon cuniculi	X	B	X	0A

NOTE: A: basic, B: optional, X: not listed.

TABLE 4 Microbiologic Monitoring Items for Rats (Bacteriology)

Item	FELASA (Europe)	Microbiological Associates (US)	ICLAS-Asia (Japan)	Score
Mycoplasma pulmonis/spp.	A	X	A	2A
Bordetella bronchiseptica	A	X	A	2A
Corynebacterium kutscheri	A	X	A	2A
Salmonella spp.	A	X	A	2A
beta-hemorrhytic streptococci	A	X	X	1A
Streptococcus pneumoniae	A	X	A	2A
Pasteurella pneumotropica	A	X	A	2A
Leptospira spp.	A	X	X	1A
Kebsiella pneumoniae	B	X	X	0A
Pseudomonas aeruginosa	B	X	A	1A
E. coli	B	X	X	0A
Proteus spp.	B	X	X	0A
Staphylococcus aureus	B	X	B	0A
Yersinia pseudotuberculosis	B	X	X	0A
Dermatophytes	B	X	B	0A
Pneumocystis carinii	B	X	B	0A

NOTE: A: basic, B: optional, X: not listed.

TABLE 5 Microbiologic Monitoring Items for Rats

Minimum Requirement	Recommended
Sendai virus	Pneumonia virus of mice
Sialodacryoadenitis virus	Mouse encephalomyelitis virus
Mycoplasma pulmonis	Kilham rat virus
Bordetella bronchiseptica	H-1 virus
Corynebacterium kutscheri	Reo 3 virus
Pasteurella pneumotropica	Hantavirus
Streptococcus pneumoniae	*Clostridium piliforme*
Salmonella spp.	beta-hemorrhytic streptococci
Arthropods	CAR bacillus
Helminths	*Leptospira* spp.
Eimeria spp.	*Pseudomonas aeruginosa*
Giardia spp.	*Klossiella* spp.
Spironucleus spp.	*Encephalitozoon cuniculi*
	Toxoplasma gondii
	Tricosomoides crassicauda
	Other flagellates

nella, arthropods, helminths, *Eimeria*, *Giardia*, and *Spironucleus*. We regard the 16 items on the right as "recommended." Similarly, we discussed monitoring items for mice and designated the 11 items on the left as "minimum" and the 17 items on the right as "recommended" (Table 6). These lists were prepared provisionally for future discussion by experts in microbiology.

CONCLUSION

Our specific proposals for the quality network include requests to breeders and reference organizations, as shown in Table 7. An adequate breeding scheme and embryo preservation for outbred stock are considered pivotal for breeders to avoid genetic drifting and backup from bottleneck. We would also like breeders to share a minimum health profile, reference substances for in-house monitoring, and ultimately establish a quality network between breeding sites or group breeders. We would like US and Japanese organizations to support our proposals by establishing an evaluation standard for genetic drifting in outbred stock, a harmonized health profile, and a list of available reference substances for validated microbiologic monitoring. I am sure that such a quality network will benefit not only humans but also the animals themselves by refining their genotype, phenotype, and dramatype.

TABLE 6 Microbiologic Monitoring Items for Mice

Minimum Requirement	Recommended
Sendai virus	Pneumonia virus of mice
Mouse hepatitis virus	Mouse encephalomyelitis virus
Mycoplasma pulmonis	Minute virus of mice
Corynebacterium kutscheri	LCM virus
Pasteurella pneumotropica	Reo 3 virus
Salmonella spp.	*Clostridium piliforme*
Arthropods	*Bordetella bronchiseptica*
Helminths	*Citrobactor freundii* 4280
Eimeria spp.	beta-hemorrhytic streptococci
Giardia spp.	*Streptococcus pneumoniae*
Spironucleus spp.	*Streptococcus moniliformis*
	CAR bacillus
	Pseudomonas aeruginosa
	Klossiella spp.
	Encephalitozoon cuniculi
	Toxoplasma gondii
	Other flagellates

TABLE 7 Proposals for Establishing a Quality Network

1. Novartis requests breeders to establish:
   - ➤ Adequate breeding scheme to avoid genetic drifting/bottleneck by generation/site
   - ➤ Embryo preservation for risk management
   - ➤ Sharing of minimum health profile
   - ➤ Sharing of reference substances for reliable screen
   - ➤ Genetic/microbiological monitoring for data-oriented quality assessment
   - ➤ Quality network between breeding sites/group breeders

2. Novartis requests reference organizations to provide:
   - ➤ Evaluation standard for genetic drifting of outbred stock
   - ➤ Harmonized minimum health profile
   - ➤ Supply of reference substances

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International Harmonization of Laboratory Animals

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STRAINS AND COLONIES USED IN ANIMAL EXPERIMENTATION

Laboratory animals may be divided into three major genetic types: inbred animals, hybrid animals, and closed colonies. Each type is used in animal experimentation in ways that maximize the application of its characteristics.

Inbred Strains

Almost no genetic differences can be found between any two animals within a particular inbred strain. Therefore, use of inbred animals generates better stability and reproducibility of results than closed colony animals in all types of animal experiments. Experiments also typically require fewer numbers of these animals, which is an important advantage with respect to animal welfare. However, because there are major genetic differences from one inbred strain to another (for example, in responses to drugs) there may be completely different results (such as a high response level in one strain and a low level in another). If an animal experiment is performed using inbred animals, it is necessary to perform the experiment first with several different strains to select the most appropriate strain.

Many types of research are also performed by utilizing strain differences in responses such as sensitivity and resistance. Examples of this research include biochemical studies on substances that cause strain differences such as proteins and enzymes and genetic studies on strain differences.

Hybrid Animals

In laboratory animal science, hybrid animals are usually obtained by mating among different inbred strains. They include the following four types: F1 hybrid, F2 hybrid, three-way cross, and four-way cross.

As explained subsequently in Genetic Test Method for Genetic Composition, genetic control of hybrids is easy, and it is possible to produce hybrid colonies with a high degree of reproducibility. Hybrids are considered appropriate for animal experiments because they generally show excellent reproductivity and good health, which compensate for the defects of their inbred parent strains such as low productivity due to inbreeding degeneration and various physiologic and biochemical defects caused by mutant genes. Historically, however, there have been few examples of the widespread use of hybrids in animal experimentation.

Closed Colonies

Closed colonies of rats and mice have long been used as representative species in experiments such as toxicity tests. Gene polymorphism is maintained in closed colonies, and the genotypes of individual animals are known to differ based on genetic testing (Katoh and others 1998). In this respect, closed colonies correspond to human populations; however, it is evident from an understanding of the origins of closed colonies that they cannot always be considered representative of species such as mice and rats. The main reason is that a single population (colony) does not possess all of the genes or gene polymorphisms of the species. There is also a strong possibility that closed colonies will lose their genetic stability because of artificial (human) control. Extreme phenomena (the bottleneck effect) concerning the number of members of colonies associated with microbiologic cleaning, in particular, are likely to occur during cesarean section, and we have experienced several actual examples of this.

SAFETY STUDIES AND LABORATORY ANIMALS

Studies in Which Animal Species Present a Problem

Mice and rats are widely used in new drug development, especially in toxicity (acute, subacute, and chronic) tests. In these studies, the responses are strong as long as the genetic differences in the same species are negligible. The doses are high, and individual differences or strain differences are not likely to appear. Although they are not performed at this time, studies formerly used the LD_{50} (50% lethal dose; the amount or concentration that causes death of 50% of the animals when a drug is administered) as the parameter. Therefore, primarily closed colonies, rather than inbred animals, have been used historically in this

type of study. In many cases, for example Wistar and Sprague-Dawley rats and ICR and Swiss mice have been used. However, these closed colonies show clear differences in spontaneous malformation rates, spontaneous cancer rates, types of cancer, body weight, and life span.

Many new drugs have been developed using closed colonies, and a large amount of data has been accumulated. When the same type of studies are performed in the future, specific consideration should be given to selection of strains based on materials used in former studies. It is natural to conclude that it is desirable to use the same strain as that used before and the time before that. Even if the strain name is the same, the name of the breeder should be reported.

These precautions are of even greater interest in high-precision research in fields such as immunology, which involves individual genes and molecular genetics. In the past, however, when the causal relation between the response and the related substance (DNA or protein) was vague or unclear, especially in typical animal experiments such as toxicity tests, almost no consideration was paid to selection of the strain.

Studies Showing Individual and Strain Differences

Animal experiments such as those performed in new drug development at present include not only those using mice and rats as typical species such as LD_{50} studies but also studies in which physiologically or metabolically active enzymes such as P450 or the p53 or *H-ras* genes are used. In these studies, it is essential to be aware of the possibility that various factors such as strain selection may affect the response because relatively weak responses are measured. The effect of the genetic background on these particular genes is unknown. In the worst case, when the genetic background is inbred, it may not be possible to perform the animal experiment. Unfortunately, however, we have had no time to evaluate the effects of genetic backgrounds of inbred animals on various genes, and we do not know whether such evaluations are worthwhile at this time.

INTERNATIONAL HARMONIZATION OF LABORATORY ANIMALS

At present, international harmonization of data from animal experiments is being promoted by the International Conference on Harmonization (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use. The most important items for animal experiments are reflected in Good Laboratory Practice. As discussed previously, the harmonization issues that have caused problems in several testing facilities have been related to laboratory animals (strains or colonies), food, and experimental methodology.

In the case of food, objective (scientific) standards can be established by identifying the contents, for example, in percentages of protein, and for experimental methods and techniques, by preparing records using photographs or imag-

ing results. For laboratory animals, however, the only objective information we currently have is that of strain names. Discussions have only recently begun to address the question of whether there is scientific evidence for a discussion on the international standardization of laboratory animals.

The need for international genetic and microbiologic standardization of laboratory animals such as mice and rats was recognized in the early 1980s. Dr. Nomura, among others, recognized that standards should be based on high-quality industrial products as well as strict methods for evaluating this quality. It was agreed that participation in the international market should be contingent on fulfillment of such standards. The results of these initial efforts toward standardization appear in the *ICLAS Manual for Genetic Monitoring of Inbred Mice* (Nomura and others 1984).

Using genetic testing before genetic monitoring, it is possible to determine the genotypes of individual inbred strains with accuracy. It is also possible to differentiate one strain from another based on whether all animals in a strain have particular genes at the gene loci or whether genotype information for several gene loci is present. This method applies not only to inbred animals but also to closed colonies. An example of this differentiation is shown in Figure 1, in which a gene of the *Es3* locus is present in 30% of colony A, 90% of colony B, and 0% of colony C.

An Ideal Global Standard

The facts and examples described above may be used as standards for a closed colony of laboratory animals. Colonies that meet these standards may be considered global standard colonies.

Items to be determined

It is possible to differentiate certain closed colonies from other colonies by the presence of particular genes in the colony or differences in the frequencies of these genes. For these reasons, the types of genes present at a certain time and their frequencies should be determined. In Table 1, the gene frequencies of 21 biochemical markers in closed colonies of the rat are shown. As is evident, *Acon1*^{a} can be detected in both SD colonies in the frequencies of 0.958 and 0.567, respectively, but cannot be detected in Wistar colonies and Donryu colony.

Prohibited items

Individual animals must not be introduced into another colony either from colonies with the same name or from colonies with different names.

Recommended items for compliance

The following methods are recommended: maintenance method (the rotation system or a system based on it is recommended to assure genetic stability);

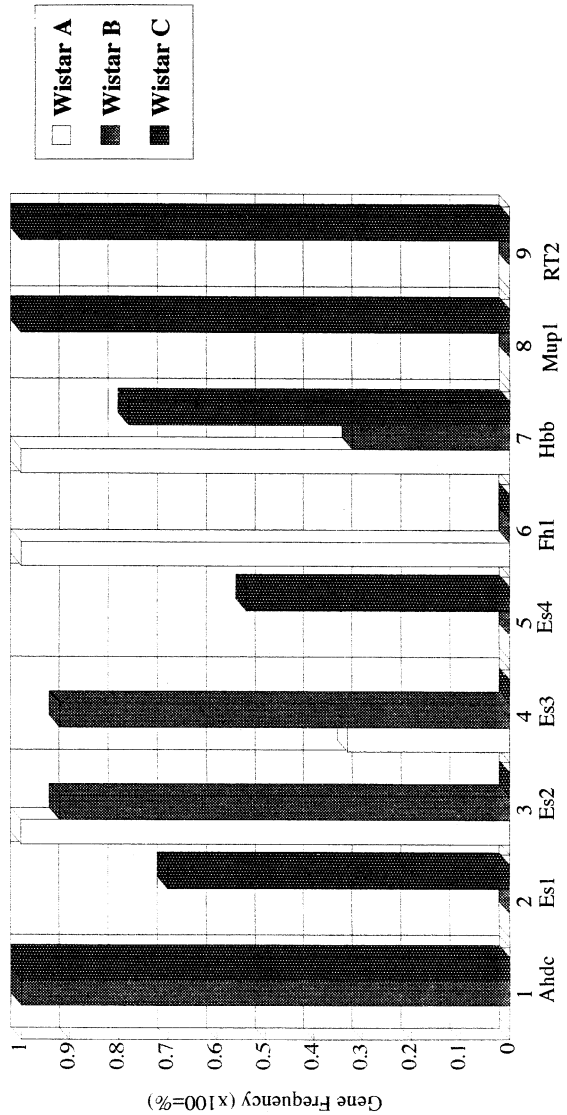


FIGURE 1 Gene frequencies of nine markers in three Wistar colonies.

TABLE 1 Gene Frequencies of 21 Biochemical Markers in Six Closed Colonies

	Locus	Allele	Jcl:Wistar	Crj:Wistar	Iar:Wistar	Jcl:SD	Crj:SD	Donryu
1	*Acon1*	*a*	0.0000	0.0000	0.0000	0.9580	0.5670	0.0000
		b	1.0000	1.0000	1.0000	0.0420	0.4330	1.0000
2	*Ahd2*	*b*	0.1830	0.0000	0.0000	0.0000	0.0000	0.0000
		c	0.8170	1.0000	1.0000	1.0000	1.0000	1.0000
3	*Ahdc*	*a*	0.0000	1.0000	1.0000	1.0000	0.9020	1.0000
		b	1.0000	0.0000	0.0000	0.0000	0.0980	0.0000
4	*Akp1*	*a*	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
		b	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
5	*Alp1*	*a*	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
		b	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
6	*Amy1*	*a*	1.0000	1.0000	1.0000	1.0000	0.6500	1.0000
		b	0.0000	0.0000	0.0000	0.0000	0.3500	0.0000
7	*Es1*	*a*	0.0000	0.0000	0.7000	0.1920	0.3750	1.0000
		b	1.0000	0.9670	0.3000	0.0000	0.1670	0.0000
		c	0.0000	0.0330	0.0000	0.8080	0.4580	0.0000
8	*Es2*	*a*	1.0000	0.9170	0.0000	0.7670	0.3080	0.0000
		b	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
		c	0.0000	0.0000	0.5420	0.1670	0.0000	0.5920
		d	0.0000	0.0830	0.4580	0.0670	0.6920	0.4080
9	*Es3*	*a*	0.3330	0.9170	0.0000	0.2920	0.3920	0.9500
		b	0.0000	0.0000	0.4250	0.0000	0.0000	0.0000
		c	0.0000	0.0000	0.2330	0.4670	0.0000	0.0000
		d	0.6670	0.0830	0.3420	0.2420	0.6080	0.0500
10	*Es4*	*a*	0.0000	0.0000	0.5420	0.1580	0.0000	0.0000
		b	1.0000	1.0000	0.4580	0.8420	1.0000	0.1420
		c	0.0000	0.0000	0.0000	0.0000	0.0000	0.8580
11	*Es6*	*a*	1.0000	0.5520	1.0000	0.4330	0.6670	1.0000
		b	0.0000	0.4480	0.0000	0.5670	0.3330	0.0000
12	*Es7*	*a*	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
		b	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
13	*Es8*	*a*	0.0000	0.9170	0.4330	0.2330	0.7170	0.1330
		b	1.0000	0.0830	0.5670	0.7670	0.2830	0.8670
14	*Es9*	*a*	1.0000	0.8330	0.5670	0.7650	0.2830	0.9150
		c	0.0000	0.1670	0.4330	0.2350	0.7170	0.0850
15	*Es10*	*a*	1.0000	0.9170	0.5670	0.7830	0.2850	0.9170
		b	0.0000	0.0830	0.4330	0.2170	0.7150	0.0830
16	*Es14*	*a*	1.0000	1.0000	0.7330	0.0000	0.5830	1.0000
		b	0.0000	0.0000	0.2670	1.0000	0.4170	0.0000
17	*Fh1*	*a*	1.0000	0.0000	0.0000	0.0250	0.0000	1.0000
		b	0.0000	1.0000	1.0000	0.9750	1.0000	0.0000
18	*Gc*	*a*	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
		b	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
19	*Hbb*	*a*	1.0000	0.3170	0.7830	0.4250	0.0000	1.0000
		b	0.0000	0.6830	0.2170	0.5750	1.0000	0.0000
20	*Mup1*	*a*	0.0000	0.0000	1.0000	0.0000	0.0350	0.9420
		b	1.0000	1.0000	0.0000	1.0000	0.9650	0.0580
21	*Svp1*	*a*	1.0000	0.5670	1.0000	0.4000	0.7170	1.0000
		b	0.0000	0.4330	0.0000	0.6000	0.2830	0.0000

long-term maintenance method (cryopreservation of embryos should be used); and measurement method of genetic variations (the gene frequency is used as an index).

Genetic test method for genetic composition

Genetic testing may be performed using the methods applied by the ICLAS Monitoring Center. These methods are recommended because a comparison between colonies is necessary.

Standard Candidates to Replace Closed Colonies

If the major problems characteristic of closed colonies are not solved, it is necessary to consider other genetic populations. Candidates are F1 and F2 hybrids as well as three- and four-way cross hybrids.

F1 hybrids

These colonies are obtained by mating between two strains. When the gene loci of A strain and B strain are expressed as A and B, A/B (heterotype) appears at all gene loci in F1 and there is no genetic difference among individuals. Because physiologic or reproductive heterosis appears, the animals show excellent traits such as active behavior or great reproductivity. However, when a genetic evaluation is performed, these colonies are considered less suitable for replacement of closed colonies with their high level of diversity.

F2 hybrids

These colonies are obtained by mating among F1 animals, and the genotype obtained is A/B×A/B. Mendel's law also applies to all gene loci in F2, namely A/A (A parent type), B/B (B parent type), and A/B (F1 type) at a ratio of 1:1:2. It is evident that the genotype frequency and gene frequency can be expressed quantitatively in F2, and the level of reproducibility of F2 is extremely high.

However, there are many genetic differences among individuals in F2 colonies, and individuals may not have exactly the same genotype. Therefore, F2 colonies have the same genetic diversity among individual animals in the colony as in closed colonies, but the level of genetic diversity is low compared with closed colonies because it is determined by the two parent strains.

Three-way cross hybrids

These colonies are obtained by mating between F1 (A/B) obtained by mating two strains (A and B) and a third C strain. The mated animals have either A/C or

B/C at all gene loci. These colonies have the same excellent reproducibility as F2, and it is possible to produce the colony as long as the parent strains are available. The genotype frequency and gene frequency can be estimated from Mendel's law.

As in F2 colonies, the same high level of genetic diversity among individual animals in the colony is present, and the genetic diversity is even greater than that of F2 because there are three parent strains. However, the extent of genetic diversity is still less than that of closed colonies.

Four-way cross hybrids

The animals are produced as shown in Figure 2: (1) Two kinds of F1 animals are produced by crossing between inbred strains (that is, line A × line B and line C × line D); (2) different F1 animals are mated, and MCH animals are produced by the four-way cross mating.

Four-way cross hybrid colonies are obtained using four strains (A, B, C and D) by mating between F1 (A/B) obtained by mating strains A and B and F1 (C/D) from mating strains C and D. This hybrid colony also has excellent reproducibility, which may be perpetuated as long as the four parent strains are available. The genotype frequency and gene frequency can also be estimated from Mendel's law.

The genotype of the gene loci of an individual is A/C, A/D, B/C, or B/D. As is evident from so many different genotypes, the genetic diversity among individual animals is greater than that in the F2 or three-way cross colonies, and this colony may be considered artificial, with the highest level of genetic diversity based on the addition of a fourth strain. However, the recessivity is greater than that of closed colonies because the gene source is limited to four strains.

In 1976, development of inbred strains was started by using 40 pairs of closed colony Jcl:ICR. At F9, they were made germ free. Crossing experiments for selection of partner strains were performed using these inbred strains, and four inbred lines were selected. Shown in Table 2 are the genetic profiles of the four inbred strains (IAI, IQI, IPI, and ICT), two F1 hybrids (IAI×IQI and IPI×ICT), and MCH obtained by crossing the F1 hybrids. Pilot production was started in 1983, and various types of basic data concerning the animals produced were collected. According to the results of the experiments performed to date, the Jcl:MCH (ICR) can be used in place of Jcl:ICR. In Figure 3, a comparison of inbred, MCH, and closed colonies is shown. MCH has the following features: (1) MCH is considered an artificial colony with the highest level of genetic diversity; however, its recessivity is greater than that of closed colonies because the gene source is limited to four strains. (2) MCH has excellent genetic reproducibility, which may be perpetuated as long as the four parent strains are available; therefore, genetic characters are balanced and remain unchanged for long periods, which leads to improved reproducibility in animal experiments. (3) Bottlenecks that currently present problems with closed colonies do not develop.

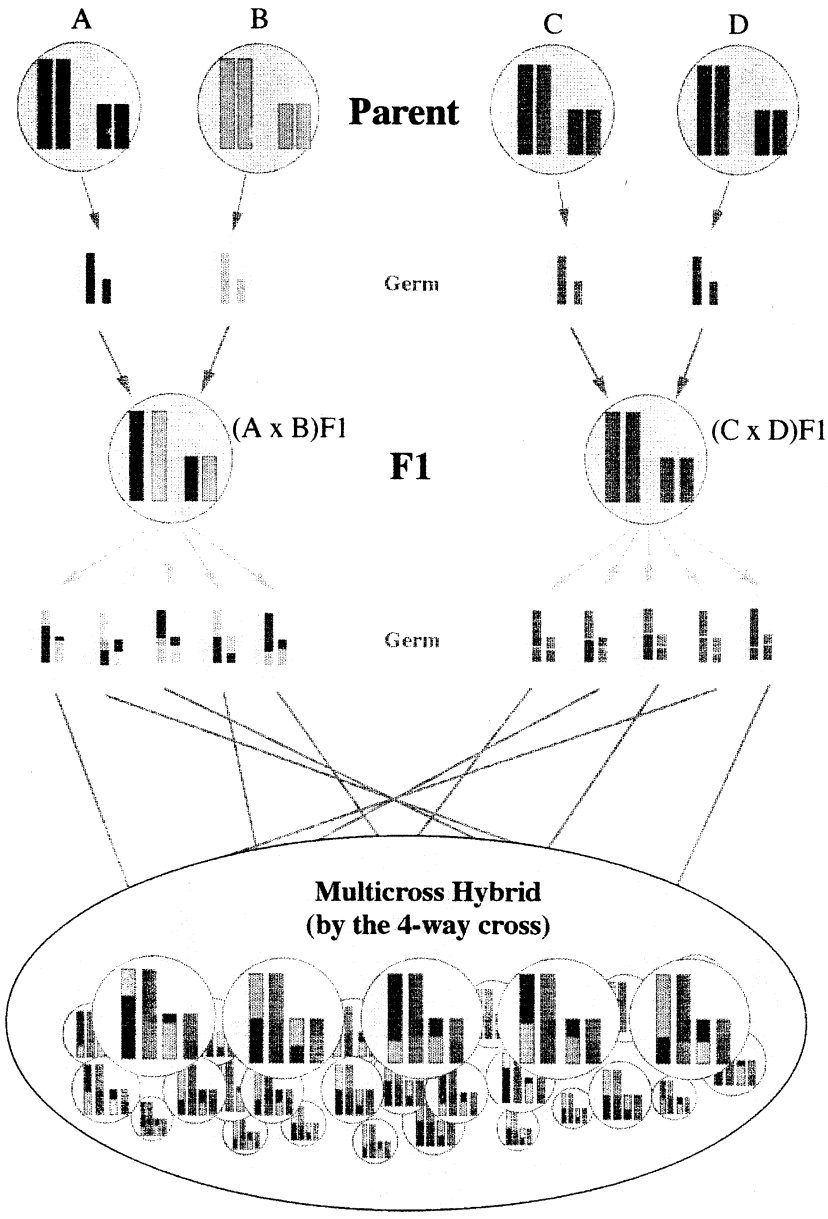
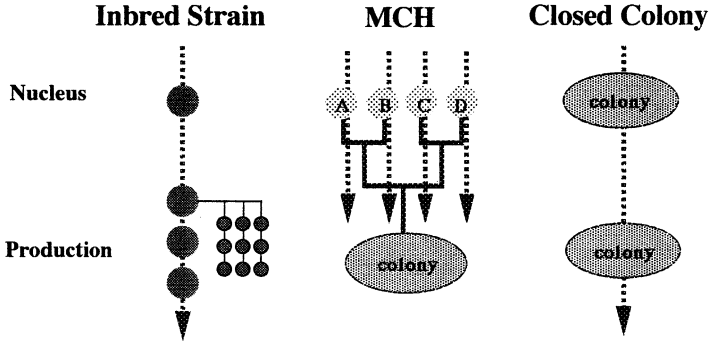


FIGURE 2 Production of the multicross hybrid by the four-way cross.

TABLE 2 Genetic Profiles of the Four Inbred Strains Derived from Jcl:ICR, F1 Hybrids, and MCH (F1 × F1)

	Marker Loci																	
Strain name	*Idh1*	*Pep3*	*Akp1*	*Hc*	*Car2*	*Mup1*	*Gpd1*	*Pgm1*	*Ldr1*	*Gpi1*	*Hbb*	*Es1*	*Es2*	*Thy1*	*Mod1*	*Trf*	*Es3*	*H2K*	*H2D*
IAI	*a*	*b*	*b*	*1*	*b*	*a*	*b*	*b*	*a*	*b*	*s*	*b*	*b*	*b*	*b*	*b*	*c*	*q*	*q*
IQI	*a*	*b*	*b*	*1*	*b*	*a*	*b*	*a*	*b*	*b*	*d*	*b*	*b*	*a*	*b*	*b*	*c*	*q*	*b*
(IAIxIQI)F1	*a*	*b*	*b*	*1*	*b*	*a*	*b*	*ab*	*ab*	*b*	*sd*	*b*	*b*	*ab*	*b*	*b*	*c*	*q*	*qb*
IPI	*a*	*b*	*b*	*1*	*b*	*a*	*b*	*b*	*b*	*a*	*d*	*b*	*b*	*b*	*b*	*b*	*c*	*q*	*b*
ICT	*a*	*b*	*b*	*1*	*b*	*a*	*b*	*a*	*a*	*a*	*s*	*b*	*b*	*b*	*b*	*b*	*c*	*q*	*b*
(IPIxICT)F1	*a*	*b*	*b*	*1*	*b*	*a*	*b*	*ab*	*ab*	*a*	*sd*	*b*	*b*	*b*	*b*	*b*	*c*	*q*	*b*
MCH(AQxPC)	*a*	*b*	*b*	*1*	*b*	*a*	*b*	*a*	*a*	*ab*	*s*	*b*	*b*	*ab*	*b*	*b*	*c*	*q*	*qb*
								ab	*ab*		*sd*			*b*					*b*
								b	*b*		*d*								



Production methods	Maintenance by sib mating	Continuous production from 4 inbred strains	Reproduction only from a colony with no animals introduced from other colonies
Reproduction	**Poor - Good**	**Excellent**	**Good**
Genetic uniformity	**Clear**	**Clear**	**Unclear**
Genetic characters	Not suitable for toxicity tests Limited range of characters	Suitable for toxicity tests Fixed range of characters	Suitable for toxicity tests Irregular variations in range of characters
Genetic reproducibility	Very good	Very good	Good
Microbiological control	**Easy**	**Easy**	**Difficult**

FIGURE 3 Characteristics of inbred strains, MCH, and closed colony.

CONCLUSION

The problems of closed colonies have recently become evident in connection with ICH. I have attempted to explain that these problems cannot be solved by looking only at closed colonies and that it is necessary to reflect on the laboratory animals currently used in animal experiments. Finally, from an overall evaluation of closed colony problems, it is clear that these problems are not problems of the animals themselves but problems related to utilization and production, that is, human problems. When these problems have been solved, it should be possible to select appropriate laboratory animals for each animal experiment.

I propose the following recommendations for solving problems related to animal experimentation. For researchers or users: Understanding (1) the limitations of closed colonies in animal experimentation (what types of experiments are appropriate and what types are inappropriate), and (2) the effects of strain differ-

ences in some experiments. For breeders: Understanding (1) the reasons quality is required for laboratory animals, (2) the necessity of using objective scientific evidence to evaluate colonies, and (3) which procedures to use to confirm the genetic quality of laboratory animals.

Finally, in animal experiments (especially safety studies) using closed colonies, I recommend collecting and preserving DNA or biochemical marker data for tests on animals (mice and rats) used in safety studies in the development process of a new drug. If the results obtained in a safety study show significant differences from those obtained in a previous study or from other institutions, the causes can be narrowed down to the following: techniques (such as method of administration), environment (such as food, including temperature and humidity), and/or animals (genetic differences between the colonies used in the studies). Techniques and environment are specified in the GLP, as mentioned; but for animals used in the studies, only the strain names remain. It is important to know the genetic composition of the individuals that comprise the colonies used in the studies if the data obtained are to be utilized effectively. I therefore recommend that the genetic test data obtained for the animals used in such studies be preserved for future reference.

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Rat Genetics and Toxicology

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SUMMARY

There is substantial genetic variation in the response of laboratory rats to xenobiotics, and this variation has important implications for toxicologic research and screening. However, most characteristics of toxicologic interest have a polygenic mode of inheritance so the variation is not immediately apparent to most investigators who use only a single stock or strain of rats.

A survey of "rat" papers published in *Toxicology and Applied Pharmacology* in 1979 and 1999 showed that the use of inbred strains has increased from 7% to 31% in the 20-year period. However, given the extensive literature on the importance of using inbred (or F1 hybrid) strains and the lack of any published scientific justification for the use of outbred stocks, this slight increase suggests that toxicologists are still not giving much thought to the most appropriate choice of animals.

Most (66%) authors failed to note the strain used either in the title or abstract, and were apparently under the impression that they were studying "the rat," even though their results gave no indication of the likely variation in response among rat strains. Only four (7%) of the 61 rat papers published in early 1999 used more than one strain, but three involved study of known genetic polymorphisms and one used two strains interchangeably. None of them would have been able to detect previously unknown genetic variation in response. Academic research workers could easily use more than one strain of rats without increasing the total numbers of animals, using factorial experimental designs. Such designs would be statistically powerful and would not present any particular statistical problems.

Given that there is substantial, and economically important, genetic variation in humans in response to xenobiotics and that rats are widely used in toxicology and pharmacology, the failure to seek genetic variation in rats, which could be used as a model of similar conditions in humans, is surprising. Unless toxicologists change their research tactics they will fail to benefit from the enormous advances currently being made in molecular genetics.

INTRODUCTION

It is now 20 years since ILAR published an excellent set of guidelines on "Laboratory Animal Management: Genetics" (ILAR 1979). These guidelines described the main types of strains and stocks then available to research workers, which included inbred strains and their derivatives such as congenic strains and recombinant inbred strains, mutants, and "stocks not genetically defined," including outbred stocks.

In a discussion of the choice of inbred strains versus outbred stocks, the guidelines suggest, "An investigator working with species for which inbred strains are available would be well advised to use them." The serious limitations of genetically undefined strains were emphasized. Although such animals may be cheaper, they will be phenotypically more variable so that larger numbers are needed, they are subject to genetic drift, and colonies with the same name from different breeders may differ to a serious extent. Moreover, since that time there have been many papers published that describe the valuable properties of inbred strains and the limitations of outbred stocks (Festing 1990, 1995, 1997a,c; Festing and Wolff 1995). Yet, outbred stocks continue to be used widely, even though no scientific justification for their continued use appears to have been published in the last 20 years.

In contrast, inbred strains, which have been described as "immortal clones of genetically identical individuals," tend to be highly uniform, they stay genetically constant for long periods, the genetic and phenotypic characteristics of most strains are well documented, and genetic quality control is relatively easy using DNA genetic markers (Festing 1997b).

FROM THE LITERATURE

Strain Differences in Response to Xenobiotics

Genetic variation in response to xenobiotics is seen most clearly as strain or stock differences, in experiments that have used more than one strain (for convenience the term *strain* will be used to indicate both inbred strains and outbred stocks). Examples include differences in response to DMBA among three rat strains in which strain COP was totally resistant to a dose of carcinogen that caused 100% mammary tumours in WF, with F344 being intermediate (Moore

and others 1988), large differences in response to 3,2'-dimethyl-4-aminobiphenyl among five rat strains with 48% prostate tumors being observed in F344, but none in the Wistar stock (Shirai and others 1990), and large differences between inbred ACI rats and an outbred Sprague-Dawley stock in response to diethylstilbestrol and neutron irradiation, with the Sprague-Dawley stock being completely resistant to the effects of DES, but reasonably sensitive to the neutron irradiation, with the converse being observed in strain ACI (Shellabarger and others 1978). Large strain differences in response to pharmaceutical agents are also frequently observed (Kacew and Festing 1996).

Such strain differences have important implications for toxicologic research and screening (Festing 1987, 1995, 1997a). Without some idea of the range of sensitivity seen among different strains, it clearly does not make much sense to characterize "the rat" on the basis of research done with a single strain that may be highly atypical. For example, some colonies of outbred Han:Wistar rats have a mutation in the *Ah* receptor, which makes them approximately 1000-fold less sensitive to the acute toxic effects of TCDD than an outbred Long-Evans stock (Pohjanvirta and others 1999; Tuomisto and others 1999). However, strain differences are of intrinsic interest because there are large differences among humans in response to xenobiotics (Evans and Relling 1999). Because rats are widely used in both the pharmacology and toxicology of drug development, knowledge of genetic variation in response would provide useful animal models of human adverse drug reactions. For example, the DA rat strain (which should *not* be called the Dark Agouti strain as the D stands for the D blood group and not for the word Dark) is widely used as a model to study the effects of genetic variation at the CYP2D locus (Vorhees and others 1999).

Survey of Papers Published in *Toxicology and Applied Pharmacology* in 1979 and 1999

To obtain more details of the way rats have been and are currently used in research, papers published in a single toxicologic journal (*Toxicology and Applied Pharmacology*) in 1979 and 1999 were studied. This is a well-respected journal that publishes mechanistic studies of the effects of xenobiotics on biologic systems, including laboratory rats. The results of a study of the first 45 papers published in 1979 and the first 61 papers published in 1999 using the laboratory rat are given in Table 1. The aim was to determine what progress has been made over the last 20 years in encouraging toxicologists to use genetically defined animals and to find out what proportion of papers included more than one strain so that the investigator would have become aware of genetic variation in the observed responses.

In 1979, only 7% of papers involved the use of inbred strains, but by 1999, this use had increased to 31%. This change is almost entirely accounted for by an increase in the use of F344 rats at the expense of Sprague-Dawleys, with the use

TABLE 1 Survey of the First 45 and the First 61 "Rat" Papers Published in *Toxicology and Applied Pharmacology* in 1979 and 1999

	1979		1999	
No. of papers		45		61
	No.	%	No.	%
Used inbreds	3	(7)	19	(31)
Used outbreds[a]	42	(93)	43	(70)
Use strain/stock				
SD	29	(64)	25	(41)
Wistar	10	(22)	12	(20)
F344	2	(4)	14	(23)
Other	5	(11)	9	(15)
Strain in title/abstract[b]	8	(18)	27	(44)
Used more than 1 strain	1	(2)	4	(7)
Able to find new genetic variation[c]	0	(0)	0	(0)

[a]One paper used both.
[b]Used as an indication of whether the author(s) considered that they were investigating "the rat" or a particular strain of rats.
[c]Papers that used more than one strain comparatively when not investigating a known polymorphism.

of Wistar and "other " rats staying approximately constant. Thus, some slight progress has been made in encouraging the use of inbred strains, although whether this rate of progress is acceptable, given the limitations of these outbred stocks, is debatable. None of the papers gave any reasons for choosing the strain used.

In 1979, only a single paper in the sample used more than one strain, and that was a study involving a known genetic polymorphism. Thus, no papers would have detected previously unknown genetic variation. By 1999, 7% (4/61) of papers in the sample used more than one strain, but three of the papers involved studies of known genetic polymorphisms, and one used two strains interchangeably in different experiments without indicating which strain had been used when presenting the results. Thus, none of the studies was in a position to observe genetic variation that was not already known.

Toxicologists often appear to assume that the strain or stock of rats they use is representative of "the rat" in general (Festing 1990). As an indicator of this assumption, the survey also recorded what proportion of the papers failed to mention the strain of rats used either in the title or in the abstract. In 1979, 18% of papers noted the strain in this way, but this proportion had increased only to 44% by 1999. Thus, more than half the papers apparently characterized "the rat" on the basis of work done with a single, often undefined strain of rats. Statements such as "The oral LD_{50} of adenine in the rat is 227 mg/kg" have very little meaning if strains can differ substantially in their response to such a xenobiotic.

Multistrain Experiments Do Not Necessarily Need to Involve the Use of More Animals

The quality of much toxicologic research could be substantially improved if toxicologists sometimes used more than one strain. Their failure to do so appears to stem from the assumption that the use of two strains would double the number of animals needed, but this assumption is wrong. In most cases, it would be entirely valid statistically to use the same number of animals, but divided among two or more strains using a factorial experimental design (Festing 1999).

There are four possible strategies with respect to genotype that could be used in studying the effects of a xenobiotic either in toxicologic research or in screening. For simplicity, it will be assumed that an experiment will involve a control and a treated group and that there will be a total of 48 rats in each group. In practice, group sizes are about this large in toxicologic screening, but there are usually three or four treated groups with different dose levels of the compound. Possible experimental designs involve the use of a single inbred strain, a single outbred stock, identical twins, or several isogenic strains but without increasing total numbers.

Single inbred strain

The first design involves the use of a single inbred strain. This design has the advantage that the treated and control groups are genetically identical at the start of the experiment, so such an experiment would tend to have high statistical power provided the strain is genetically susceptible to the compound. However, if the strain is unusually resistant, then this strategy will not be very good and, as it only uses a single strain, the experiment will not indicate whether the response is under genetic control.

Single outbred stock

The second design involves the use of a single outbred stock, as is currently most common. This design has four serious limitations: (1) If the outbred stock is genetically heterogeneous, then the treated and control group will not be genetically identical at the start of the experiment. This genetic difference will normally lead to increased phenotypic variability so that the experiment will lack statistical power. (2) The stock may, like a single inbred strain, be genetically resistant to the xenobiotic. (3) The experiment may not be repeatable elsewhere because outbred stocks with the same name often differ; and (4) Because individual genotypes or pedigrees are unknown, there will be no indication that the response is under genetic control.

Identical twins

The third design involves the use of 48 pairs of identical twins (assuming they are available). It is well established that twin experiments in humans and

cattle are extremely powerful because, on the one hand, the treated and control groups are genetically identical, and, on the other hand, the differences between twin pairs will sample a broad range of genetic variation in susceptibility. In humans, such an experiment could, in theory, include people of different racial groups to sample a wide range of human genetic polymorphism. Differences between twin pairs will give some indication of the range of genetic variation present in the human population, although a formal test is not possible unless twin pairs could be stratified, for example, by race or on the basis of some known genetic polymorphism. Notice that with twin studies, there are no particularly difficult statistical problems in analyzing the data. The group size for comparing treated with control groups is the same as if a single group had been used, although for quantitative characters, a paired rather than an unpaired *t*-test would be used. For qualitative characters, the total number of responders would be compared in the treated and control groups across the whole experiment. Thus, this design would, in theory, be very good, although in practice, identical twin rats are not available, and it would be inconvenient to use two rats from each of 48 strains.

Several isogenic strains, but without increasing total numbers

The fourth design is a suitable compromise between the use of a single isogenic strain and the use of twins or 48 isogenic strains. Thus, the experiment could consist of small numbers of several different strains. For example, instead of using 48 rats of a single strain, it would be possible to use, say, 12 rats of each of four isogenic strains. Strains could be chosen at random, on the basis of known susceptibility to the class of agent being studied, or to be as genetically diverse as possible. This design has the advantage that the treated and control groups are genetically identical at the start of the experiment, and the differences between the strains will sample a range of genetic variation in susceptibility. In many ways, it is very like the twin study and presents no particular problems for statistical analysis. Toxicologists sometimes mistakenly see this as four separate experiments, each of which is too small; however, given that with a twin study or when using an outbred stock it is quite permissible to average across genotypes, there is no statistical reason why the same should not be done with this design. The more strains that are used, the more statistically powerful the experiment becomes (Felton and Gaylor 1989). As the differences between inbred strains are usually quite large (which is a feature of the effects of inbreeding [Falconer 1981]), it is in some ways rather like a twin study that was able to sample different racial groups, making it quite a powerful design. This design could be used immediately by academic toxicologists who are not under the same regulatory constraints as those doing toxicologic screening for commercial purposes. In the long term, such a design could also be used in regulatory toxicology once it has been used in academic work and its useful properties have been explored in some detail.

CONCLUSIONS

Toxicologists continue to use genetically undefined outbred stocks, although the case for using inbred or F1 hybrid strains has been made repeatedly in the past, and has never been seriously criticized. Moreover, very few academic papers surveyed involved more than one strain, so toxicologists are often not aware that the responses they observe may differ to a considerable extent in a different strain.

The use of a multistrain experiment as part of a series of experiments involving the study of toxic mechanisms would alert toxicologists to the importance of genetic variation. Some investigators would then be able to start using modern tools of molecular genetics which would almost certainly lead to a better understanding of toxic mechanisms. However, until toxicologists start to use isogenic strains and begin to compare several strains as a routine part of their research, most of them will continue to be stuck in the dark ages as far as genetics is concerned.

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A Phenotype-driven Approach to the Molecular and Functional Analysis of the Mouse Genome

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BACKGROUND

The mouse genetics and mutagenesis program at Oak Ridge National Laboratory (ORNL) is employing chemical mutagenesis and broad-based phenotype screening to recover mutations targeted to specific chromosome regions for ascertaining the whole organism functions of mouse genes. Our strategy of chemical mutagenesis results in pedigrees of mice, each harboring a different DNA mutation for one of the many genes contained in the chromosome region. These mice are then subjected to a broad range of tests to identify a mutant phenotype. This phenotype-driven approach is being applied initially to about 8% of the mouse genome and is adaptable to any genome region as the necessary genetic resources are developed. However, the efficiency with which we can discover genes using this strategy hinges on our proficiency in detecting abnormal phenotypes in the progeny of mutagenized mice. To this end, we have designed broad-based, high-throughput screening assays that are performed on multiple animals from the same pedigree to identify obvious or subtle aberrations in behavior, biochemistry, and/or morphology in mice at young and old ages. Eventually, DNA sequence will be coregistered with functional information for each gene using mouse mutations as the gene-discovery tools and the phenotypes those mutations specify as indicators of gene function.

The mouse genetics program at ORNL began in 1947 under the direction of Dr. William L. Russell. Since then, experimental mutagenesis has been performed mostly to assess genetic risk from exposure to a variety of radiations and

chemicals and has been focused on seven specific loci (Russell 1951) that generated visible phenotypes when mutated. In the mid-1970s, Dr. Russell discovered that the chemical *N*-ethyl-*N*-nitrosourea (ENU) is a supermutagen for mouse spermatogonial stem cells (Russell and others 1979), inducing primarily point mutations (single base pair substitutions) (Russell and Montgomery 1982) and thus a variety of types of mutations including nulls and hypomorphic alleles (Bedell and others 1996; Ji and others 1999; Marker and others 1997). In 1986, one of us (E.M.R) launched a pilot ENU-mutagenesis experiment (Rinchik and Carpenter 1999) focused at the albino (*c*; now called *Tyr* [tyrosinase]) locus in mouse chromosome (Chr) 7. This region is covered by an extensive series of radiation-induced deletion mutations resulting from the Russell specific locus tests. The mutagenesis strategy for the *Tyr* region, adapted from a similar approach used by *Drosophila* geneticists, was to mate ENU-mutagenized males (BALB/cRl) that are homozygous for the *c* coat-color marker to wild-type females (Rinchik and Carpenter 1999). The F_1 mice bearing the BALB/c Chr 7 carrying newly induced point mutations, some of which will be closely linked to *c*, were then mated to carriers of a large "selector" deletion at *c*. Of the progeny from this second mating, 25% should be albino and may also express an additional new mutant phenotype if an ENU mutation is so closely linked to *c* that it maps within the limits of the large *c* deletion. A set of simple complementation crosses to smaller *c* deletions localized new mutant phenotypes to intervals suitable for a positional-cloning approach (Rinchik and Carpenter 1993, 1999; Rinchik and others 1993). A similar experiment for the deletion complex surrounding the pink-eyed dilution (*p*) locus, also on Chr 7, is currently under way (Johnson and others 1995; Rinchik and others 1995; Rinchik, Carpenter, and Johnson, manuscript in preparation). Our successes in designing the genetics and logistics of large mutagenesis experiments have led to the establishment of our current program of inducing new mutations in the proximal two thirds of Chr 7 (which includes the *p* and *c* regions), the central one half of Chr 10, the distal half of Chr 15, and a small segment of the X chromosome.

In all of these experiments, broad-based screening for the detection of new mutant phenotypes plays a new and very prominent role. The current program has benefited from our hands-on experience as we increase both the chromosomal region target sizes and our scope and capacity for examining mice for as many different kinds of abnormalities as possible. Our current experiments take advantage of deletion screens, as described above, and more powerful methods utilizing chromosomal inversions as tools to make newly mutagenized chromosomes homozygous—all without molecular genotyping.

EXPERIENCE APPLIED TO NEW EXPERIMENTS

We have learned three important lessons from pilot ENU experiments that have influenced the much broader mutagenesis and phenotype-screening pro-

grams now under way. These lessons relate to mutation recovery rate, visual genotyping, and the importance of genetic reagents for targeting mutagenesis.

Mutation Recovery Rate

In the pilot experiment at *c*, using the 6- to 11-cM deletion c^{26DVT} (Rinchik and Carpenter 1999) as the "selector deletion," only visible and lethal phenotypes were ascertained. Even so, 31 new mutations were recovered in 4557 gametes (pedigrees) screened for a mutation recovery rate of one in 147 pedigrees tested (Rinchik and Carpenter 1999). We know that mutagenesis and phenotype screening within different regions will result in the recovery of more or fewer mutations due to gene density differences in the regions, and/or to the proportion of genes in the regions that can mutate to a visible or lethal phenotype. Thus, whereas gene density is fixed, we should be able to affect mutation recovery rate by expanding the number and kinds of phenotype screens to increase the proportion of genes for which we can detect a mutation.

Visual Genotyping

Having the progeny class that carries no wild-type copy of the newly induced mutation (i.e., "test class" mice that have one deletion chromosome and one mutagenized chromosome marked by carrying a visible marker like *c* or that is homozygous for the mutagenized chromosome) provides several important advantages: (1) It eliminates the need for molecular genotyping, an expensive, error-prone, and logistically difficult procedure. (2) It permits 100% ascertainment of lethals, evident when the visibly marked "test" class is absent in progeny or does not survive as long as other genotypes. In our pilot experiments, about half of all new mutations are lethals, which would go undetected if progeny genotypes could not be distinguished by external phenotype. (3) It allows the easy production and testing of multiple test class animals, all carrying the same mutagenized chromosome, for assay in tests with highly variable parameters (such as behavioral tests) or testing in multiple sites. In our program, four test class progeny from each pedigree go through phenotype screening, with the requirement that all four show the variant phenotype before being designated "mutant" and bred for transmissibility of the trait. (4) It likewise allows for the shelving of a set of test class progeny for aging and retesting for late onset recessive mutant phenotypes.

Importance of Genetic Reagents for Targeting Mutagenesis

Although the *Tyr*- and *p*-region experiments have been quite successful, it is clear that chromosomal deletions are too small to be used exclusively as selectors for new mutations genome-wide. Deletions are clearly indispensable for comple-

mentation crosses to localize new phenotypes within the genome region of interest but generally cannot be large enough (due to the negative effects of large haploinsufficiencies) for efficient genome-wide mutagenesis. Chromosomal inversions, on the other hand, can be larger, and cover a much larger region of chromosome. When suitably marked for visual genotyping (Rinchik 2000), inversions also suppress recombination events that might separate the new mutation from the linked visible "tracking" marker. Accompanied by overlapping sets of nested chromosomal deletions generated in vitro using molecular techniques for the modification of embryonic stem cell chromosomes (Ramirez-Solis and others 1995; Thomas and others 1998; You and others 1997), large inversions become ideal tools for regional mutagenesis. As the mouse and human DNA sequences are acquired and analyzed for these mutagenized regions, we can begin to integrate physical and functional gene maps.

ROBUST AND BROAD-SPECTRUM PHENOTYPE SCREENING

Overview

Over the past 2 years, we have employed a basic set of primary phenotype-screening tests to enhance our mutation-recovery rate by detecting mutant phenotypes that are not apparent upon routine observation of test class mice. We have recently expanded and updated our instrumentation to automate our screening as much as possible to accommodate the anticipated load from new and ongoing experiments. Furthermore, all pedigrees will be rescreened at 18 months of age to ascertain later onset abnormalities. Tests in current use have been validated by testing mice that we expected to show a mutant phenotype; we have also used these tests to screen nearly 2000 mice on a high-throughput basis and have identified new mutant phenotypes that would have escaped detection with our previous methods. We are accumulating and testing new equipment fairly constantly and replacing older or less efficient instrumentation as we can. The primary screen is the only opportunity to detect subtle anomalies, so it is crucial that this screen be comprehensive, well-grounded, practical, reliable, and capable of performing as promised.

Because we do have multiple test class animals in every pedigree to screen, we can rely on replication of any abnormality; and with heritability testing, false positives are minimized, even with highly variable traits. We are also certain, by visual genotyping, that we are performing our screens only on test class mice that can be expected to exhibit recessive mutant phenotypes, thus maximizing throughput and minimizing cost. Comprehensive screening, realistically designed, gives us greater power to discover mutant phenotypes of interest that historically have slipped through the cracks. We have planned our screening so that whenever possible, more than one assessment tool will target each category of mutant phenotype of interest to minimize further both false positives and false negatives.

Behavioral Tests

In the establishment of a comprehensive set of phenotype screens, we have chosen tests that are reliable, practical, easily automated, and likely to detect phenotypes of interest to neuroscientists. Individual behavioral tests are rarely definitive measures of a single neurologic process. However, by employing multiple tests, we can develop a pattern of response across tasks to inform us about a particular process. For example, a mouse with heightened anxiety but no memory deficit may perform poorly in the Y-maze spontaneous alternation memory task but show improved performance in the conditioned freezing memory task. However, a mouse with an actual learning deficit should perform poorly on both tests, allowing us to be more confident that the impairment is one of memory and not a nonmnemonic process. In a similar manner, we can dissect sensory and motor components of an aberrant behavior while still employing a test set that can actually be accomplished in an efficient and high-throughput manner.

For each pedigree generated from most mutagenesis experiments, four test class mice (usually two males and two females) first go through the weaning screen at 21 to 25 days of age (P21-25), next the primary screen at P50-60, and then are aged along with two additional males and two additional females for rescreening at 18 months (P548-560). Two pairs of the mice are mated for fertility testing and later separated for storage or sent for further testing if sterility is observed in either sex. Mice are examined at weaning (P21-25) for visible aberrations (overall size and proportionality, external genitalia, limbs, digits and tails, eyes and ears, fur color and quality, posture and gait). The remaining tests are performed over 3 days, in order from the least aversive to the most aversive to avoid intertest impact. Mice rest for at least 1 hour between tests, and all testing is done between 8:00 a.m. and 4:00 p.m. We test approximately 100 mice per week at P50-60 and later retest them at 18 months (P548-560). If recessive visible or lethal phenotypes are apparent, breeding stocks are established without further primary screening. The tests currently used to identify alterations in these traits are outlined in Table 1.

The general flow of mice, when they are 50 days of age and again at 18 months of age, through the behavioral/central nervous system screen is described below. The process is designed to minimize interest impact.

Day 1

Each mouse is weighed, and simple, gross neurologic observations are performed (reaching reflex, vibrissae response, righting reflex). Next is the 2-minute rotorod test (Accuscan SmartRod, Columbus, Ohio[1]) for balance and

[1]Identification of commercial products or manufacturers does not constitute an endorsement by the authors or the National Research Council.

TABLE 1 Primary Behavioral/Central Nervous System Screening Tests

Assessment	Age	Method
Observation	P21-25, P548-560	Observation for physical or behavioral abnormalities by trained staff
Vibrissae placing	P50	Head turn in response to stimulation of the whiskers by a pen tip
Reaching reflex	P50-60, P548-560	Lower mice toward flat surface and observe for reaching response
Vision	P50-60, P548-560	Pupillary reflex in response to light
Hearing	P50-60, P548-560	ICR click box; pinna response noted
Nociception	P50-60, P548-560	1. Toe pinch 2. Tail-flick meter for latency to respond to heat stimulus
Locomotor activity and rearing	P50-60, P548-560	1. Activity recorded by photobeams in the open field 2. Polytrack video recording of open-field movement
Motor co-ordination	P50-60, P548-560	Rotorod latency to fall, 4-minute acceleration test
Learning/memory	P50-60, P548-560	Cued/contextual conditioned freezing, 4-chamber inserts for motor activity system
Startle response Sensorimotor gating Habituation	P50-60, P548-560	Startle magnitude, habituation to startle, and prepulse inhibition assessed in a single session.
Anatomy/morphology	P50-60, P548-560	MicroCT scanner, 1 mouse per pedigree

coordination, designed to measure ability to maintain position on an accelerating rotating dowel. Four mice at a time are then videotaped for 3 minutes (preceded by a 2-minute habituation period) in individual 24" × 24" opaque chambers open at the top. Observed behaviors (amount and patterns of movement) are analyzed using software accompanying the Polytrack Video System (San Diego Instruments, San Diego, California) to automatically compute relative dwell time for each user-defined portion of the open-field chamber. This is followed by assessment of acoustic startle response, prepulse inhibition of startle (PPI), habituation to startle in a single session in an automated system (Hamilton-Kinder Co., Poway, California), and return to the home cage.

Day 2

Open-field activity is measured by counting interruptions of a set of photobeams over a 20-minute test period to provide information on exploratory and motor behaviors (Hamilton-Kinder). Rearings are monitored, as are total activity count (beams broken) and locations of beam breaks within the enclosure. Next is the tail-flick test, in which latency to react to a heat stimulus is measured by

confining the tail of the mouse in a slot and timing the latency to tail withdrawal after heat is applied; the mouse is removed within 10 seconds if it does not appear to perceive the heat. Last on Day 2 is the conditioned-freezing training trial. The open-field activity system includes 9" × 9" × 9" opaque white plexiglas inserts that fit into the photobeam frame; the bottom of the insert is an electric grid. Mice are lifted into the insert, confined with an opaque lid, and allowed to acclimate for 2.5 minutes. They are then presented with an 85-dB tone (3000 Hz) for 30 seconds and then a 2-second 0.4-mA footshock, followed by a 2-minute recording of posttone/postshock activity. The mouse is then removed to its home cage.

Day 3

The first test is the 24-hour conditioned-freezing memory retention trial, in which the mouse is returned to the same chamber that was used for the training trial and allowed to explore for 3 minutes while its movement is monitored to detect freezing behavior. The mouse is removed to the home cage, and the chamber context is changed by insertion of a black liner. The mouse is returned for a 2-minute free exploration period, followed by repetition of the 30-second tone (no shock). Movement is again monitored for 3 minutes to detect freezing. Data are in the form of the total count of photobeam breaks in the first-day free exploration (presound and shock) compared with photobeam breaks the second day for either the 3-minute context test or the 3-minute postsound cue test. By this method of analysis (that is, comparing the mouse with itself in the training vs. retention trials), correction is made for the innate activity level of the individual mouse strain.

Biochemical, Physiologic, and Molecular Tests

Blood, urine, and various tissue samples are harvested from test class mice for a broad array of biochemical tests. Numerous tissues from one male and one female from each pedigree are taken at P60 and frozen for future primary or secondary screens. In this way, tissues can be analyzed retrospectively as additional genome/complex pathway information becomes available. Sperm will also be cryopreserved in the event that later recovery of the pedigree through in vitro fertilization is required. Using fluid or tissue samples from the test class allows efficient use of test class mice inasmuch as samples from any mouse can feed a large number of tests; we also archive a variety of tissues from each pedigree for future primary or secondary screens.

Tests in current use as primary screens for all pedigrees include characterizations of 12 hematologic parameters using a Cell-dyne 3500 Hematology Analyzer (Santa Clara, California) and measurement of six factors in urine using a Bili-Labstix dipstick (Bayer Corp., Elkhart, Indiana). We have recently acquired

the capability to measure blood levels of glucose, cholesterol, and triglycerides and will begin immediately to acquire these values for all test class mice.

Anatomy/Morphology

ORNL engineers have developed a small animal computed axial tomography X-ray scanner (MicroCT; Oak Ridge National Laboratory, Oak Ridge, Tennessee) with image reconstruction software and rudimentary organ recognition software for use in phenotype screening. This instrument takes a whole-body scan at less than 1-mm resolution in about 6 minutes and can resolve both soft tissues and skeleton. It is currently equipped with kidney recognition algorithms, and algorithms are under development for lean body mass and whole body fat content determination. Significant skeletal malformations (such as scoliosis) will also be detected from examination of these reconstructed and stored CT images. One test class mouse anesthetized by isoflurane is imaged from each pedigree, with all images stored on CD-ROM.

Tennessee Mouse Genome Consortium (TMGC)

ORNL is a charter member of the newly formed TMGC, a statewide organization designed for enhanced phenotype screening and analysis. Clinical and academic experts from the University of Tennessee Medical Center at Memphis, the University of Tennessee at Knoxville, Vanderbilt University, St. Jude's Children's Research Hospital, the University of Memphis, and Meharry Medical College have joined forces to screen ORNL test class mice comprehensively for a wide variety of phenotypes. Early efforts have concentrated on phenotyping for abnormalities in the nervous system (behavior, drug sensitivity, neuroanatomy, sensory organs, neurochemical pathways, sleep/wake cycles) and are now expanding into heart, blood, and lung phenotypes. Domain experts will also continue phenotype analysis once a mutation is identified. Because ORNL can generate multiple animals per pedigree, live mice or samples from mice can travel statewide (or farther) for primary and secondary screening programs that greatly increase our opportunities for realizing the highest possible mutation recovery rate from our mutagenesis program.

Statistical Analysis and Flagging Mutant Mice

We use the test results from all other test class animals from the same mutagenesis experiment as controls inasmuch as all mice are handled the same and the majority of pedigrees yield normal test class progeny for any given phenotype. This allows us to establish a criterion of two standard deviations from the mean from a very large population, giving us quite adequate statistical power with which to flag a mutant pedigree while testing only four (or even fewer) mice

from that pedigree. If at least three of the four tested show a deviant phenotype, we send that pedigree for transmissibility testing. In our experience, all four will show the variant phenotype, but we take note that we do have a segregating genetic background that could affect the phenotype in any one animal. Potential mutants are first tested for transmissibility of the trait. If the trait proves heritable, then several things can occur:

1. a breeding stock is established;
2. the stock can be rederived so that secondary screening of interest can be done in an SPF facility if appropriate, inasmuch as our current colony is conventional;
3. test class mice are sent to interested researchers for confirmation and more detailed secondary/tertiary screening and analysis of the phenotype and gene-cloning; and
4. all primary data are currently entered into Excel spreadsheets, and from there will become part of a laboratory information system under development by ORNL's Computational Biosciences/Bioinformatics group.

SUMMARY

The goal of this large-scale mouse mutagenesis and phenotype screening program is to annotate DNA sequence with experimentally derived functional information about how individual genes perform in the context of a whole mammalian organism. We have initially undertaken mutagenesis of about 8% of the mouse genome, distributed in different genome regions depending on the current availability of appropriate genetic resources. Resource building continues as a fundamental part of this program to facilitate the application of this strategy to the rest of the mouse genome. Expansion and enhancement of our phenotype screening capabilities are integral components of the entire effort as we continue to extend our capabilities in the detection of all abnormalities that mutant mouse genes can exhibit.

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QUESTIONS AND ANSWERS

DR. DELL: Do you perform open field testing during the day or night? I ask because the animals are active 90% of the time at night but only 10% of the time during the day. So your observation period could be shorter under infrared at night.

DR. JOHNSON: Under infrared, I am sure that is true. The logistics of setting up infrared in our colony would simply be prohibitive. I would remind

you that we are measuring all pedigrees against the other thousand pedigrees that have had the same test. We have already found animals that are two standard deviations above and two standard deviations below the population mean. Maybe if we were screening at night we would be finding a whole different set of mutants; however, it is not possible to do everything.

DR. FESTING: Please clarify your statement that you mutagenize the males. How many generations do you have to go through before you get the test animals?

DR. JOHNSON: Our pilot experiments generated data from more than 2000 animals using two-generation hemizygosity screens. In the second generation, the animal carries the mutagenized chromosome opposite a chromosome deletion. That experiment is very simple and effective. It greatly limits the size target in the genome because deletions can be only a certain size before there are haploinsufficiency problems. Some genome regions will not tolerate deletion at all.

We now are doing large chromosomal inversions, which require three generations. In the third generation before you have brought the mutagenized chromosomes to homozygosity, you can make as many animals as you want. Making more animals in that third generation so that you can breed it to make a fourth generation of multiple test class animals will limit the number of overall pedigrees that you can screen, which you do not want to do because only a certain percentage of the pedigrees are mutants in a region, or are mutant to a phenotype.

DR. JACOB: Please describe the average size of the deletion and whether there is a set amount.

DR. JOHNSON: The size of the deletion is very region dependent. We have two deletions that are up to about 6 cM each (such as the ones I described in the *p* region), and we have some in other regions that are probably 10 or 11 cM. We will not be able to make them much larger than that. For example, there is an imprinted region in the distal end of the *p* region that has the Prader-Willi/Angelman syndromes in humans, and it is not possible to make a deletion there in males.

DR. MORIWAKI: What kind of mutation is in that region?

DR. JOHNSON: At this time, we are simply mating two of the animals to learn whether they are fertile. If it is male sterile or female sterile, then we know people who are interested in taking that mutant for analysis. Again, this is primary screening. We are only saying that this animal is infertile; we do not know why. Those animals are advertised on our Web site and are available for anyone who is interested. Ours is a conventional facility. We been successful in mailing blastocysts overnight and having people transfer them into clean animals for rederivation of the stock.

Evaluation of Targeted Mutations

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Our mission at Pfizer is different from the one Dr. Johnson described in that we most often are trying to look at targeted mutations. We have tried to combine our internal expertise to be able to phenotype genetically altered mice. I think if you survey the literature for genetically altered mice, you will find that often phenotyping is inadequate or incomplete. We have attempted to avoid repeating errors of the past by formulating a systematic team approach toward phenotyping.

The necessary speed of analysis in this very competitive industry and the complexity of the science involved in both the production and analysis of these mice have been the two primary driving forces in the development of this team approach. The approach has also been the outcome of a deliberate increase in the working relationship between our discovery group; our core genetic facility that produces transgenic, knock-in, and knock-out mice; our pathologists; and our laboratory animal clinicians, who are responsible for the care of these mice.

A typical core team consists of a principal investigator (a biologist from any area in drug discovery), one or more molecular biologists (responsible for making the constructs), a pathologist and/or clinical pathologist (depending on the genetic alteration), a laboratory animal clinician, and other specialists (depending on the gene being targeted). For these teams to work quickly and efficiently, they must be able to communicate effectively. Each member must maintain a basic understanding of the technologies used in creating these mice. Team members speak with each other because technologies will always affect phenotype. Keeping up with all of the technologies being produced is becoming increasingly a challenge.

In the beginning, we had only transgenic technology, which affected the philogenetic background of the mice (quite rarely on FNB). In addition, posi-

tional effects and even insertional mutagenesis produced phenotypic surprises quite often. With knock-in and knock-out mice, we were able to avoid those surprises, but positional effects, as with transgenic mice, are present throughout the ontogeny of the animal. So we still must deal with the problem of embryonic and fetal lethals.

Today we have a whole array of tissue-specific gene promoters, heterologous recombination systems. These systems allow us to avoid many of the developmental problems, but they create new problems. Many of the inducible systems, for instance, use drugs such as tetracycline or dexamethasone, which can produce effects in bone or in the thymus, both in the morphology and in the function.

For our mouse plan to progress smoothly, we develop our phenotyping plan in advance. When possible, we want to customize each plan to fit the particular mouse project and the circumstances. We try to encourage the principal investigators to assemble all of the team members to solicit their input so that we are assured of having the required reagents and of identifying the necessary techniques. With this approach, we also can begin breeding and husbandry plans, ensuring that we have adequate numbers of mice for later analysis. Finally, we make every effort to utilize the best scientific practices for obtaining accurate phenotypes.

We customize the phenotyping plans because we often want very targeted, specific models. We want to be able to focus the evaluations, to narrow the scope of the evaluations to identify the critical determinants of that model. Then, as soon as a mouse is produced, we confirm the functional success of the alteration by molecular phenotyping. Our methods of choice are, as usual, Northern blots or reverse transcription polymerase chain reaction. For our primary characterization of changes in gene expression, we use Western blots and enzyme-linked immunosorbent assay primarily for analyzing the changes in protein expression. These methods are non-slide based. The more advanced ones we use are in situ hybridization and immunohistochemistry, which is slide based and performed by a pathologist. We are increasingly finding that gene microarrays are useful when the team is interested in determining which secondary changes have been induced by the targeted genetic alteration.

We complete the molecular phenotyping before we schedule any additional analysis. We follow our primary molecular phenotyping with pathology, including gross examination pathology and clinical pathology in most of these plans. For a full validation of any phenotype, we perform a comprehensive pathology evaluation so that we can identify anticipated as well as unanticipated changes in phenotype. Often the secondary changes will render these models useless for us.

Timing is an important aspect of the evaluations. We first try to determine the timing for evaluation of the mice rationally, and if that is not possible, we use clinical signs. If we are not able to determine the best time for evaluation, we use a periodic default timing, which targets puberty, adulthood, and full maturity, as

needed, to characterize the phenotype. When we are looking for targeted models, we perform full gross examinations. We select the organs we will evaluate for routine histology. If it is intended to be a model for Alzheimer's disease, we will look (perhaps only) at the brain. We may include major organs if those are not among the target organs. Then we select either routine or special clinical chemistry and hematology tests, again, depending on our knowledge of the targeted gene.

Once the model has been shown to have the critical determinants, we proceed to the comprehensive pathology evaluation. In instances in which the purpose of the analysis is simply to characterize the phenotype (which it often is), when we are exploring the function of a newly discovered gene, we will go directly into comprehensive pathology. We then perform full gross examination to obtain histopathology on a full set (about 40) tissues. We run a routine clinical chemistry panel, hematology, and other special tests as needed, such as hormones or cytokines, again depending on the target gene.

The best scientific practices for accurate phenotyping require experienced molecular laboratories. Laboratories should be accustomed to working with RNA and particularly the immunohistochemistry that now utilizes custom antibodies for analyzing these mice, which can often pose particular challenges.

Pathologists who perform the analysis should also be experienced in rodent pathology and in situ electrotechniques. It is necessary to understand the genetic background of these mice and be familiar with the spontaneous as well as age-related lesions that can occur. Pathologists must also be able to trouble shoot the in situ techniques and understand the procedures and the common problems that can arise. We often encounter embryonic or fetal lethals in which cases we use specialists (either developmental biologists or pathologists) who are trained in murine development. Finally, we always insist on adequate sample sizes, appropriate control mice, and an environment controlled for feed, light, and housing (particularly if they are models for the study of cancer or endocrinology).

Sometimes teams can be very awkward vehicles for solving problems. However, we have found that at Pfizer, we are able to phenotype mice quickly, while making the best use of our resources, by working in teams. For us, working in teams is really the only way to proceed.

Defining Behavioral Phenotypes in Transgenic and Knockout Mice

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INTRODUCTION

Experimental manipulation of the mouse genome provides a powerful new technology to generate animal models of human genetic disorders. A well-defined phenotype of the mouse model can serve as a quantitative, robust surrogate marker to evaluate the efficacy of potential treatments for the human disease.

Neuropsychiatric illnesses generally present as a complex set of symptoms. Multiple genes contribute to primary causes and to susceptibility factors. Symptoms are often cyclical and may vary with age and level of neurodegeneration. Biologic and environmental components interact in determining the etiology of the disease. Targeted gene mutation mouse models can be useful in parceling out each of the genetic components of the disease.

More than 100 transgenic and knockout mice with mutations in genes expressed in the nervous system have been generated to date (Picciotto 1999). Aberrant behavioral phenotypes have been documented in many lines of mice with mutations in genes expressed in the central nervous system. Mouse models of neuropsychiatric disorders mimic the human behavioral symptoms to a greater or lesser extent in targeted gene mutations relevant to Alzheimer's disease, Huntington's disease, Parkinson's disease, amyotrophic lateral sclerosis, ataxia, epilepsy, generalized anxiety, schizophrenia, and obesity (Bauer and others 1999; Bedell and others 1997; Burright and others 1997; Campbell and Gold 1996; Contarino and others 1999; Gingrich and Roder 1998; Jucker and Ingram 1997; Klockgether and Evert 1998; Lee and others 1996; Nelson and Young 1998; Picciotto 1999; Price and Sisodia 1998; Smithies 1993; Wahlsten 1999).

To define the precise behavioral concomitants of the genetic manipulation, our laboratory has been addressing methodologic issues for the behavioral phenotyping of mutant mice. Guidelines based on strategies that have proven successful in studying a variety of new transgenic and knockout mice in our laboratory and others are extensively described in recent publications (Crawley 1999, 2000; Crawley and Paylor, 1997; Crawley and others 1997a,b; Picciotta 1999; Rogers and others 1997; Silver 1995; Wehner and Silva 1996). This discussion highlights the critical features of existing guidelines.

CRITICAL ISSUES PRECEDING BEHAVIORAL PHENOTYPING

Breeding Strategy

Two or more strains are often used to develop the set of mice for behavioral experiments. The 129/SvJ or another 129 substrain is used for the embryonic stem cells. C57BL/6J or another inbred or outbred strain is used for the blastocyst donors. C57BL/6J or an outbred strain is often used for the breeders. Varying ratios of the genetic backgrounds from each strain will be present in each offspring. Background genes from each parent may have profound effects on behavioral tests. Unknown interactions between the mutated gene and the varying background genes will compromise the interpretation of the behavioral phenotype of the mutation.

Congenic breeding of the mutation into the chosen inbred strain for seven generations will create a uniform genetic background, reduce unknown gene product interactions, and reduce variability due to random assortment of parental alleles (Crawley and others 1997a; Picciotto, 1999; Silver 1995; Wehner and Silva 1996). Suggestions for optimizing the choice of inbred strain for breeding the mutation are reviewed in Crawley and others (1997a). C57BL/6J is a strain that breeds relatively well and shows average scores on many behavioral tasks, allowing detection of both increases and decreases in the behavioral scores in a mutant line bred onto a C57BL/6J background.

Number of Mice Needed for Behavioral Phenotyping

One extreme individual can dramatically skew the results of a pilot experiment with small Ns. Larger numbers of mice are required for behavioral experiments than for many other phenotypic assays. To obtain statistically meaningful results, most behavioral experiments require 10 to 20 mice per treatment group. The treatment groups are the homozygous mutants (–/–), heterozygous mutant littermates (+/–), and wild-type littermates (+/+). If sex differences are detected, N = 10-20 for each sex of each genotype is required. Ages of the mice must be approximately the same across treatment groups. Adult mice at ages 3 to 8 months are relatively homogeneous on most behavioral tasks.

If large numbers of animals are not available simultaneously, experiments can be repeated with small groups as litters become available. All three geno-

types must be represented within each set of experiments. Data across repeated experiments can be combined if no differences are detected between the wild-type controls across the dates of testing.

BEHAVIORAL TESTS

Characterization of the behavioral phenotype is best conducted in progressive stages (Crawley 1999, 2000; Crawley and Paylor 1997; Paylor and others 1998). A four-step characterization for behavioral evaluation of a new mutant mouse line is recommended based on the experiences of our laboratory in the behavioral phenotyping of over 25 transgenic and knockout mouse lines.

Initial Observations

The first stage is a set of preliminary observations to evaluate overall health (Crawley and Paylor 1997; Paylor and others 1998). A general examination of the mice is conducted in the home cages. Any gross abnormalities in overall health, home cage nesting, sleeping, feeding, grooming, and condition of the fur are noted. Body weight and body temperature are measured. Any unusual patterns of locomotion, hyperreactivity to handling, or fighting in the home cage are recorded. Abnormal appearances and home cage behaviors provide important clues for subsequent experiments to define the behavioral phenotype. Ataxias and seizures are often first detected in the home cage (Brennan and others 1997). Aggressive behaviors in nitric oxide synthase knockout mice were first detected by animal caretakers who reported fighting in the home cage (Nelson and others 1995). Absence of normal huddled sleeping patterns in the home cages led to the discovery of social interaction abnormalities in *dishevelled-1* knockout mice (Lijam and others 1997).

Evaluation of Neurologic Reflexes

Several quick tests reveal debilitating neurologic and physiologic problems (Crawley and Paylor 1997; Paylor and others 1998). The righting reflex is a simple test in which the mouse is turned onto its back; the time it takes for the mouse to right itself onto all four paws is measured. Eye blink reflex occurs when the cornea is approached with a cotton tip swab. Ear twitch reflex occurs when the ear is touched with a cotton tip swab, resulting in immediate movement of the pinna. The whisker-orienting reflex is observed by touching the vibrissae on one side; the whiskers stop moving and the head turns to the side on which the whiskers were touched.

Sensory and Motor Abilities

Quantitative measures of sensory functions and motor skills are obtained with several tasks. Many are of short (such as 5-minute) duration. Most require

specialized equipment. The better tests for acuity in evaluating vision, hearing, and smell require sophisticated neurophysiologic recording equipment or complex operant discrimination tasks.

Hearing

Gross hearing ability is assessed by the acoustic startle response. A mouse will flinch in response to a sudden loud sound. Acoustic startle to a loud tone is quantitated by an automated startle system that measures amplitude of whole body flinch (Davis and others 1982). Sensitive measures of hearing acuity are conducted with neurophysiologic recording from the auditory nerve using the auditory brainstem response (Erway and others 1993).

Vision

A visual cliff detects blindness. The visual cliff response is quantitated in a box with a horizontal surface and a vertical wall drop-off that represents a ledge (Fox 1965). The inner horizontal surface of the box and vertical drop-off are covered with black and white checkerboard contact paper, which emphasizes the cliff-like drop-off. A piece of clear Plexiglas spans the ledge so that there is no actual drop-off but only the appearance of a cliff. The mouse is placed on a platform at the border between the horizontal surface and the vertical drop-off. Normal mice will step down mostly onto the horizontal surface to avoid the cliff they see on the other side of the platform. Blind mice, not seeing the apparent cliff, will step down an equal number of times onto the horizontal surface and the cliff-like drop-off. This test is compromised by the ability of normal mice to use sensory feedback from the whiskers and feet for edge detection. Another simple test of gross visual ability is the latency for a mouse to enter a dark area when the mouse is placed in a brightly lit area. Because mice are nocturnal and prefer the dark, a mouse with normal light/dark perception will quickly enter the darkened chamber. A blind mouse will have a much longer latency to enter the darkened chamber.

More sensitive measures of visual acuity are obtained with tasks that require training, using visual stimuli in a conditioned reward paradigm. Neurophysiologic recording from the optic nerve or the visual cortex during presentation of visual stimuli will yield the most precise measures of visual acuity.

Smell and taste

A simple test for olfactory anosmia is failure to retrieve a buried food source. A simple test for taste insensitivity is failure to avoid water flavored with quinine. However, the quick versions of these sensory tests have not been well characterized in mice and are influenced by motivational factors. Sensitive measures of

smell or taste require training on operant discrimination tasks with graded olfactory or gustatory stimuli (Ackroff and Sclafani 1998). Components of learning and memory may confound a purely sensory interpretation of operant discrimination tasks.

Accurate measures of olfactory acuity are obtained with neurophysiologic recording from the olfactory epithelium or olfactory cortex in response to odorants (Belluscio and others 1998). Accurate measures of gustation are obtained by neurophysiologic recording from the chorda tympani branch of the facial nerve in response to lingual application of taste stimuli (Wong and others 1996).

Touch

Sense of touch is evaluated by the reflexive twitch response to Von Frey hairs, fine wires of graded thickness touched to the paw (Pitcher and others 1999). Pain sensitivity is measured by the latency to lick or lift a hindpaw in the hot plate test, or to move the tail out of the path of an intense light beam in the tail-flick test (Matthes and others 1996; Sora and others 1997).

Motor

Open field exploratory locomotion is the most common measure of general motor abilities. Open field activity is measured with a photocell-equipped automated apparatus that quantitates locomotion and rearings in an empty open field (Pierce and Kalivas 1997). Coordination and balance are quantitated on the rotarod, consisting of a precisely accelerating rotating cylinder (Carter and others 1999; Lalonde and others 1996). The ability of the mouse to climb up or down a pole, and to walk along a narrow beam, represent additional measures of balance and coordination (Carter and others 1999; Paylor and others 1998). Measurement of the ability of the mouse to hang from a wire by its paws provides an index of neuromuscular strength (Paylor and others 1998). Footprint pathway analysis to quantitate abnormal gait is conducted by videotaping locomotion in a Plexiglas tunnel, or by dipping the hindpaws in black ink and allowing the mouse to walk across white paper through a tunnel (Barlow and others 1996; Carter and others 1999; Clarke and Still 1999). These several tests detect major abnormalities in spinal motor neurons and cerebellum.

Hypothesis Testing

Specific behavioral tasks are then designed to test hypotheses about the function of the gene and to model the symptoms of the human genetic disease. Relevant behavioral phenotypes are often discovered during sensory and motor analyses. The auditory brainstem response detects impaired acoustic acuity in mice (Erway and others 1993) and can be used to analyze hearing in mice with

deafness candidate genes (Robertson and others 1997). A mutant mouse model of Tay-Sachs and Sandhoff diseases, deficient in the hexosaminidase enzyme that degrades gangliosides, shows neuronal ganglioside accumulation and concomitant progressive decline in performance on the rotarod task (Sango and others 1995), analogous to the motor deficits that characterize this human syndrome. *Atm* knockout mice, a model of ataxia telangiectasia, are impaired on the open field and rotarod tests and show unusual footprint patterns (Barlow and others 1996), analogous to the ataxia seen in the clinical syndrome.

To investigate genes with unknown functions, the experimental design often requires several hypotheses and a thorough knowledge of the tests available in the existing behavioral neuroscience literature. Genes expressed primarily in the cerebellum would be investigated in tasks that measure motor coordination and motor learning. Genes expressed primarily in the hypothalamus would be investigated in tasks including feeding, stress responses, and reproductive behaviors. Genes expressed in the hippocampus and cortex would be tested in learning, memory, and attentional and habituation tasks. Genes expressed in the mesocorticolimbic dopamine pathway would be investigated in motivational, appetitively rewarded, stressor, and drug abuse paradigms. Genes expressed in the periaqueductal grey and dorsal horn of the spinal cord would be investigated in pain threshold tests and for responses to analgesics.

Many good behavioral tests are available for each of the behavioral domains of interest. Reviews cited above describe specific tests and reference the source literature for methodologic details.

Learning and memory tests for mice include spatial navigation learning tasks such as the Morris water task, Barnes maze, radial maze, T-maze, and Y-maze; rewarded tasks such as nose-poke for a food reward in an operant chamber or a five-hole chamber on various schedules; and aversive tasks such as passive avoidance, cued and contextual conditioning, and taste aversion. These tasks have been applied to the behavioral phenotyping of a variety of transgenic mouse models of Alzheimer's disease (Hsiao and others 1996) and mutations in signaling genes (Silva and others 1997). Knockouts of genes expressed in the hippocampus and regulating neuronal calcium-related signaling show deficits in learning and memory tasks (Cho and others 1998; Impey and others, 1998; Mayford and others 1996). **Feeding** tests include 24-hour consumption, limited daily access, macronutrient sources, taste discrimination, and sham feeding. Some of these tasks have been applied to study genes regulating feeding and obesity (Huszar and others 1997; Pelleymounter and others 1995). **Reproductive** behaviors are quantitated by standardized scoring of sexual activity in male mice, lordosis response in female mice, and parental latency to retrieve pups to the nest and to nurse, groom, and nest with the pups. Estrogen receptor knockout mice are impaired on sexual behaviors (Rissman and others, 1997). Oxytocin-deficient mice fail to lactate (Nishimori and others 1996; Young and others 1996). Good models of **anxiety**-related behaviors include the elevated plus maze, the

elevated zero maze, light↔dark exploratory transitions, and the Vogel conflict test. Corticotropin-releasing factor transgenics and knockouts for genes expressed in the amygdala show unusual anxiety-related behaviors on stress-related tasks (Contarino and others 1999; Heinrichs and others 1997). **Drug abuse** tendencies can be measured with conditioned place preference, two-bottle choices, and intravenous self-administration. Opiate receptor knockout mice are aberrant on tests for pain responsivity, analgesic effects of morphine, morphine withdrawal responses, and conditioned place preference (Matthes and others 1996; Sora and others 1997).

Order of Testing

Our laboratory recommends an order of testing that begins with the home cage observations, continues with observations of general health and neurologic reflexes, then addresses sensory and motor abilities, and finally focuses on the behavioral domains relevant to the specific hypotheses. This approach allows the investigator to detect underlying physiologic abnormalities in the mutant mice that might limit their ability to perform the procedures necessary for complex behavioral tasks. False positives are prevented, which would have been caused by artifacts such as blindness limiting performance in a visual discrimination learning task, hearing and olfactory deficits responsible for poor parental pup retrieval, or ataxias impeding elevated plus maze arm entries. Instead, the hypothesis-driven tests are designed to accommodate the physical limitations of the mice. For example, an auditory tone cue instead of a visual light cue is used in the automated operant chamber in a learning task for blind mice.

To avoid false negatives, our laboratory recommends choosing three or more tasks within the behavioral domain of interest. Different types of memory, different types of anxiety, different components of feeding, different types of parental care, different symptoms relevant to schizophrenia and so forth may be differentially regulated by the gene of interest. Spreading a wider net allows the investigator to catch the particular type of phenotype relevant to the mutated gene. Choice of multiple tasks is further based on differing sensory modalities and motor requirements. For example, three complimentary memory tasks would include cued and contextual conditioning (employing auditory and olfactory cues, with minimal motor requirements), the Morris water task (spatial navigation with visual cues, swimming, and stress components), and taste aversion (gustatory cues, long retention time). If deficits in learning and memory are detected in all three tasks, the findings are likely to be biologically meaningful and highly replicable. If deficits in learning and memory are detected in only one or two tasks, that type of cognitive function is further explored. For example, a deficit only on the Morris water task would be further explored with other spatial navigation tasks such as the Barnes maze and the radial arm maze.

In some cases, combinations of tests cannot be conducted in the same mouse. Interference between tasks often becomes an issue when two tasks are very

similar. Passive avoidance is similar to cued and contextual fear conditioning and to light´dark exploration. These tasks require the mouse to remember sensory associations with a dark chamber or a location where a footshock was previously received. These three tests are best conducted with different sets of mice. Alternatively, it may be possible to conduct related tests in the same mice with sufficient intervention between the tests.

Carryover effects limit some combinations of tests in the same individuals. Repeated testing in the Digiscan open field induces habituation to the novelty of the open field environment. Stressful tasks such as the Morris water task will affect performance on sensitive anxiety tests and are therefore best administered as the last behavioral assay. Similarly, drug treatments should be administered at the end of the behavioral phenotyping series. Some drugs are slowly metabolized, such that residual drug remains in the mouse for several days. Other drugs induce sensitization or tolerance to repeated doses and to doses of other drugs in the same class. Past treatments with amphetamine and cocaine induce sensitization to the effects of an acute dose of these psychostimulants on hyperlocomotion and dopamine release (White and Kalivas 1999). Repeated treatments with neuroleptics and D1 antagonists result in increased catalepsy scores (Chinen and Frussa-Filho 1999).

Getting Started

Molecular geneticists planning to begin behavioral phenotyping experiments are encouraged to develop collaborations with reputable behavioral neuroscientists. Correct choices and implementation of behavioral tasks in mice require knowledge of more than 50 years of scientific literature in behavioral neuroscience, understanding of the standard methods for the basic behavioral paradigms, and familiarity with the technical tricks that make any method work well. Experience with proper testing and handling of mice, to minimize stress factors and to meet the international guidelines for the care and use of laboratory rodents, can best be gained by spending some time working in an established behavioral neuroscience laboratory. Entering into a scientific collaboration with a recognized behavioral laboratory will help to avoid artifacts, generate statistically and biologically meaningful data, and complete behavioral phenotyping experiments with the maximum speed and precision.

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Defining Phenotype in Genetically Engineered Mice

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INTRODUCTION

Genetically engineered mice have become popular tools in recent biomedical research. However, only a few among thousands of genetically engineered mice so far reported have been established as laboratory animals that have controlled quality and are being produced and used on a large scale. The aims of defining phenotype in genetically engineered mice are as follows: (1) to define clearly the difference between genetically engineered animals produced for the purpose of elucidating the function of a gene or genes in vivo and laboratory animals used as a tool for studying the mechanism of diseases or testing drugs in vivo; and (2) to define clearly the difference among "genotype," "phenotype," and "dramatype." Dramatype, which is an altered function of the organism induced by changes in the environment and phenomena seen in the disease state, is the most important characteristic of laboratory animals.

HISTORICAL DEVELOPMENT

The following two examples of genetically engineered mice have been developed as useful laboratory animals in the biomedical field: the TgPVR21 mouse (poliovirus receptor transgenic mouse) and the *ras* H2 mouse (human proto-*ras* transgenic mouse).

TgPVR21 Mouse (Poliovirus Receptor Transgenic Mouse)

1990-1991: Establishment of human poliovirus receptor transgenic mice
1991-1993: Establishment as a laboratory animal (standardization of characters and successful large-scale production) and development of methods for neurovirulence testing and safety assurance by castration (Levenbook and Nomura 1997)
1993-1995: World Health Organization (WHO) collaborative study of TgPVR21, 1st phase
1995-1997: WHO collaborative study of TgPVR21, 2nd phase
1997-1999: WHO collaborative study of TgPVR21, 3rd phase
1999: Approval of neurovirulence test of oral poliovirus vaccine alternative for monkeys by WHO Expert Committee on Biological Standardization

ras H2 Mouse (Human Proto-*ras* Transgenic Mouse)

1988: Establishment of human proto-*ras* gene transgenic mouse
1990-1992: Backcrossing and establishment of congenics
1992-1996: Validation study for carcinogenicity testing in Japan (Yamamoto and others 1998)
1996-1999: Validation study for rapid carcinogenicity testing in the United States, the European Union, and Japan

CONCLUSIONS

As shown above, establishment of a novel laboratory animal from a genetically engineered animal is a lengthy process and requires many steps as follows: (1) establishing of a genetically engineered animal; (2) phenotyping and selection of candidate animals; (3) study of functions in purpose-oriented environments or experimentation (dramatyping); and (4) establishment of a human disease model as a laboratory animal.

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Development of the Mouse Model Dramatype for Human Clinical Benefit

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Human patients suffer from various diseases that are affected by multiple factors. In Figure 1, this "dramatype" is depicted from a physician's point of view. The dramatype not only includes "phenotype" but is also affected by several extrinsic factors represented by the proximate environment (further explained in Dr. Nomura's discussion in this volume). Here the dramatype is the patients' symptoms, which are caused by a combination of several factors including genetic background, developmental environment, and proximate environment such as current social environment and medical care. In many cases, the proximate environment plays the major role after birth.

Physicians should see each patient as an individual. Simply looking at the disease itself is not enough, because each patient has a different genetic background, different developmental environment, and different proximate environment, which are not described in a textbook. Physicians should also see the patient as a whole person. Because the final goal of biomedical research is to treat and prevent *human* diseases, we must always take all of these factors into account. Thus, defining the phenotype of the mouse model should be done in the context of defining dramatype.

In patients with so-called "smooth brain," or classical lissencephaly, the brain surface is basically smooth and without the usual cortical folds, which are important in normal brains to increase the surface area dramatically. Several mouse models share some of the characteristics of this disease. For example, in the *reeler* (Falconer 1951; reviewed in de Rouvroit and Goffinet 1998) and *yotari* (Yoneshima and others 1997) mutant mice, the cerebellum is much smaller than normal and lacks the foliated structure ("smooth cerebellum"). Although these

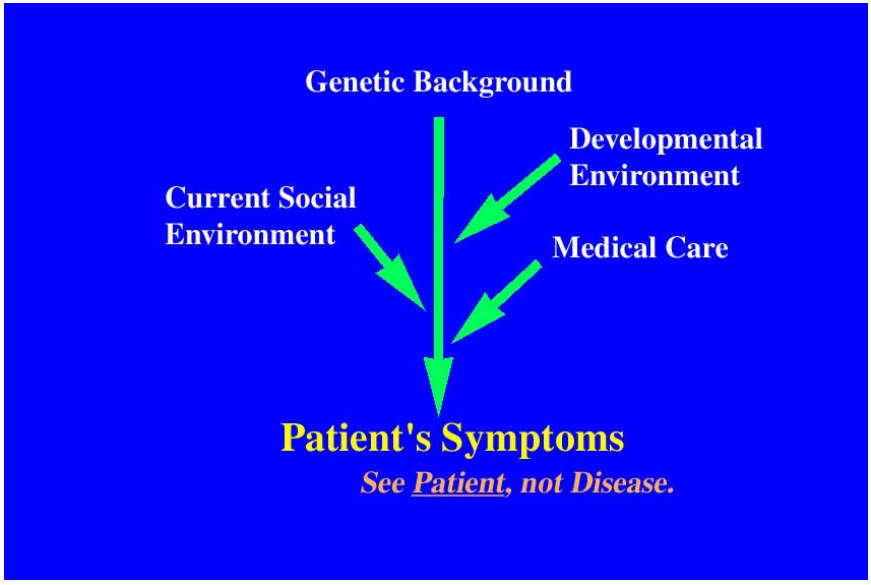

FIGURE 1 Clinical symptoms comprise human patients' "dramatype," which includes genetic background, developmental environment, and proximate environment.

mutants do not exactly correspond to the human lissencephaly, the mechanism of the "fold" formation is nevertheless likely to be shared in some way among them. Our ultimate goals in conducting experiments with these animal models are to prevent and find a cure for this terrible disease.

The *reeler* is a well-known mutant mouse found approximately half a century ago. In the *reeler*, the neocortical structure is basically inverted because of abnormal neuronal migration and the mouse exhibits ataxic gait and tremor. The *yotari*, a novel mutant mouse we found unexpectedly a few years ago, has a phenotype almost identical to that of the *reeler*. As with humans, we started from the phenotype and took an unusual approach.

We immunized *reeler* mutants with homogenates of normal embryonic brains (Ogawa and others 1995). We expected the immunized animal to produce an antibody against a molecule present only in the normal brain but not in the mutant brain. This approach worked well, and we did obtain an antibody that recognized cells in the margin of the brain in the normal mouse but not in the *reeler* mouse. This antibody, CR-50, became a very useful tool because it inhibits the function of the antigen (del Rio and others 1997; Miyata and others 1997; Nakajima and others 1997; Ogawa and others 1995). The antigen recognized by this antibody was identified by a couple of groups and was named Reelin (D'Arcangelo and others 1995; Hirotsune and others 1995). It instructs the migrating neuroblasts to

be aligned appropriately. However, the yotari mouse has a mutation in the *dab1* gene (Kojima and others 2000), which is expressed in the migrating neuroblasts (Sheldon and others 1997). We learned that the *reelin* and *dab1* genes act on a common signaling pathway, in which the reelin signal is mediated by Dab1 to control neuronal migration (Howell and others 1999; Rice and others 1998).

A completely unexpected breakthrough occurred recently in this field when Joachim Herz, who was working on lipoprotein metabolism, made double knockout (KO) mice of the very low density lipoprotein receptor (*VLDLR*) gene and apolipoprotein E receptor 2 (*ApoER2*) gene and found that the mice's phenotype was similar to that of the *reeler* mouse (Trommsdorff and others 1999). It was then learned that Reelin binds to these receptors to tranduce the signal to the intracellular Dab1 protein (D'Arcangelo and others 1999; Hiesberger and others 1999). This information was surprising not only for the developmental neurobiologists but also for those working on lipid metabolism, and it reflects the importance of interaction between completely different fields. To define the phenotype of only one mouse ("my" mouse) is not enough. New discoveries most likely depend on combining data of various mice in different fields, although practically it is not always easy.

Now that I have explained some of the genetic background of a particular diseased state in mice caused by abnormal neuronal migration, I would like to focus on the extrinsic (environmental) factor. Schizophrenia is a severe disorder that affects about 1% of the whole population. Recent reports have indicated abnormal neuronal alignment in schizophrenic brains, which hints at neurodevelopmental causes for this disease. Also, *reelin* expression has been shown to be significantly reduced (approximately 50%) in schizophrenic patients (Impagnatiello and others 1998). The neurodevelopmental etiology of this disease has also been suggested by epidemiologic reports that indicate an association between maternal second trimester infection of influenza virus and increased risk of later development of schizophrenia (Wright and others 1995; Figure 2). Recently, Dr. S. H. Fatemi (University of Minnesota) infected pregnant mice with human influenza virus at midgestation and analyzed the neonatal brains. Interestingly, he found abnormal neuronal migration associated with reduced *reelin* expression (Fatemi and others 1999; Figure 2). Although the abnormal pattern of neuronal migration was not identical to that observed in the *reeler* mouse, this study implicates the role of an extrinsic factor for affecting the Reelin-mediated neuronal alignment.

We do not yet know whether and how Reelin is involved in the development of schizophrenia; however, one possibility is that the infected mice have produced an antibody against the viral antigen, which cross-reacts with a molecule essential for the cortical development. In addition, it is possible that multiple genetic factors (such as *reelin*, *dab1*, *VLDLR*, and *ApoER2*) and environmental factors affect the final pattern of neuronal alignment through a common molecular pathway (as seen in Figure 1).

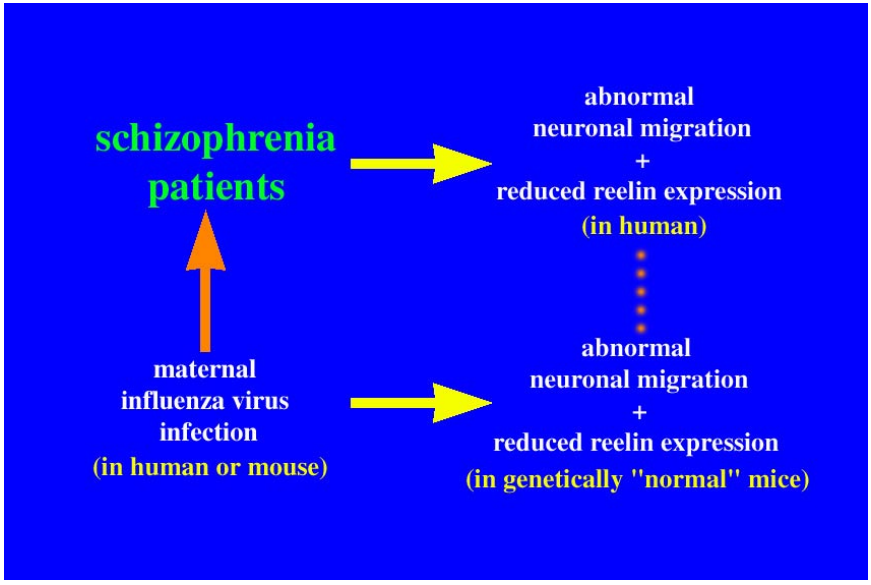

FIGURE 2 An abnormal neuronal migration pattern is hypothetically combined with reduced *reelin* expression to produce an antibody against the viral antigen, resulting in the development of schizophrenia.

In the context of dramatype development, it will be important to know at which step (genetic background, developmental environment, or proximate environment) each factor (or gene of interest) is involved because our ultimate goal is to overcome the dramatype (patient symptoms) by controlling all of these factors.

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Concluding Remarks

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I simply want to mention what I perceive to be the fundamental difference in microbiologic management in the United States, Europe, and Japan. In Japan, where laboratory animal science began in the 1950s, most if not all microbiologists are involved in research on infections and immunity. From the beginning, we have been concerned about microbiologic quality. In the United States and Europe, however, the focus is on health surveillance or health monitoring, which is very different. Microbiologic quality covers good health, but health monitoring does not cover microbiologic quality.

Recently, Japanese molecular geneticists have requested very sophisticated animals for the analysis of gene expression related to immunity or infection. They need animals with very high microbiologic quality, and we cannot compromise. For instance, we simply never use animals with an inapparent infection until the infection has been eliminated.

In Japan, we have only one ICLAS Monitoring Center (CIEA), which selects minimum requirements; and for 20 years, we have had no problems. Of course, we are prepared to encounter problems, and so we continue to exchange information and ideas frequently.

Japanese universities maintain good cooperation because the Ministry of Education supports all the university animal centers, and they all have in-house microbiologic laboratories. If a problem develops, they immediately send it to the center. I believe we are fortunate to have this simple system, which is different from US and European systems.

Finally, on behalf of the Japanese, I would like to thank the US hosts and all of the staff for organizing this excellent meeting. We, the Japanese, have learned much.

Implication of Wild-derived Genes, Mitochondria, and Chromosomes in the Genetic Background of Mouse Models for Diseases and Biologic Functions

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INTRODUCTION

Asian wild mouse-derived genes, mitochondria, and chromosomes are useful in the development of mouse models for biologic functions and their abnormal forms, diseases. Medical geneticists have recently recognized the effect of multiple genes on various phenotypes expressed at the whole body level. Obviously experimental analysis of these genes to include programmed mating can hardly be achieved in humans; therefore, it is advantageous to use experimental animals and, in particular, laboratory mice. For precise gene mapping in mice, several thousands of recently developed microsatellite DNA markers play an important role. However, due to limited progenitors, genetic variations detected among the current laboratory strains are rather limited. We need genetic resources with more genetic variations than those within conventional laboratory mouse strains.

GENETIC POSITION OF ASIAN WILD MICE

Since 1975, we have surveyed genetic variations in the natural populations of wild mice collected from all over the world. Taxonomically those mice have been classified into 11 subspecies based on their morphologic characters and geographic distribution (Schwarz and Schwarz 1943). However, our survey in the chromosome C-band pattern (Moriwaki and others 1985), biochemical markers (Bonhomme and others 1984; Moriwaki and others 1979), mitochondria DNA (Yonekawa and others 1981) ribosomal DNA (Suzuki and others 1986), and other genetic characters (Moriwaki and others 1986) has suggested the possibility

that those 11 subspecies can be further grouped into four subspecies groups as follows: domesticus, bactrianus, castaneus, and musculus (Moriwaki 1994; Moriwaki and others 1990). Genetic divergence time among them has been estimated to be approximately one million years (Moriwaki and others 1979; Yonekawa and others 1981). In these studies, the genetic origin of the laboratory mice was identified as mostly European wild mice (Yonekawa and others 1982). The implication of this finding is correct, that more genetic variations should be found between the Asian wild mice and laboratory strains. The percentage of variation in the microsatellite DNA polymorphism between the Asian mice and laboratory mice was significantly greater than within laboratory mouse strains (95% vs. 48%) (Shiroishi and others, unpublished data).

USE OF THE ASIAN WILD-DERIVED RECOMBINATION HOST-SPOT GENE FOR SURVEYING NOVEL GENES THAT DETERMINE SUSCEPTIBILITY TO DIABETES

In 1982, Shiroishi and colleagues discovered a remarkable recombination hot-spot in a Japanese wild-derived major histocompatibility complex (MHC) chromosome. The frequency of meiotic recombination in the MHC chromosome with this hot-spot is more than 100 times greater than normal (Shiroishi and others 1982). Hattori and others (1999) introduced this hot-spot chromosome segment into the NOD diabetes model mouse and obtained various recombinants in the MHC region. Their comparison between the incidence of diabetes and the introduced chromosome segments indicates the possible presence of three genes at 5' upstream of the H2- K-I region, already reported to be important in the control of diabetes.

USE OF ASIAN WILD-DERIVED MITOCHONDRIA FOR STUDYING MOUSE BEHAVIOR

In 1995, Kaneda and colleagues developed mitochondria congenic strains that carry mitochondria of either the Asian wild-derived *Mus musculus* musculus subspecies or the European wild-derived *Mus spretus* species (Kaneda and others 1995). Both are genetically quite remote from laboratory mouse strains. In 1998, Nagao and colleagues demonstrated decreased physical performance of the congenic strains with a mismatch between the nuclear and mitochondrial genome, that is, the genetic background (Nagao and others 1998).

USE OF ASIAN WILD-DERIVED CHROMOSOMES FOR DEVELOPING NEW CONSOMIC MOUSE STRAINS

In 1999, Shiroishi and others (unpublished) attempted to develop new inter-subspecific consomic strains. Each of the 19 autosomes, X and Y chromosomes

of the Japanese wild-derived MSM strain, were introduced into C57Bl/6J mice by repeated back-crosses. In each of the heterozygotes, donor chromosomes without recombination were selected by several microsatellite DNA markers on each chromosome. Soon they expect to establish a set of CONSOMIC strains, which will be very useful for rapidly surveying one or more unknown gene/genes in a mutant mouse that expresses the phenotype at the whole body level. If any phenotypic difference is found between the donor and recipient strains and it is controlled by a single gene, it will be possible to readily identify the chromosome responsible for the phenotypic difference because these CONSOMIC strains prevent "noise" from genetic backgrounds. These strains are also useful in identifying a modifier gene in the genetic background because the individual with modified phenotype is mated with all of the consomic strains.

The several cases mentioned above are typical examples of the usefulness of Asian wild-derived mice in the analyses of gene/genes in the genetic background.

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Concluding Comments

John Strandberg
National Center for Research Resources
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My summary will be very brief because my review will cover the broad range of topics discussed during this ambitious program. We started by addressing the issue of microbiologic testing, and Dr. Shek recommended standardization by constant improvement, rather than setting up a regulatory-based mechanism to achieve microbiologic testing standards. He gave good reasons for doing this, including the continuing recognition of new agents as well as new methods for diagnosis.

Dr. Riley, talking about the standardization of tests, outlined a very exciting program in which they are now engaged in a first phase. This program uses standard specimens to determine the capability or accuracy of individual laboratories and to develop standard operating procedures, which I believe have been needed for a long time. From my perspective, this development is a very positive and important step.

Dr. Itoh then discussed factors that cause a lack of uniformity in results. His discussion was based on encounters in the EQUEST monitoring center with discrepancies between several specific agents such as *Pasteurella pneumotropica* and hemorrhagic fever with renal syndrome. He recommended that in harmonizing the tests, the methods must be enumerated along with the recommendations for their usefulness.

Dr. Shibahara described laboratories in Japan, which include 53 national university animal centers plus centers in private universities and municipalities and prefectures. He outlined a concern about international transfer of transgenic and knockout mice, which complicates the worldwide situation considerably. He noted that animals rejected for entry into Japanese laboratories because of infec-

tions they carried have comprised about 8% of both domestic and international introductions.

Following these presentations were additional discussions on the desirability of the establishment of recommendations by regulatory organizations, rather than just letting standards evolve over time.

Dr. Katiyama pointed out the need to share minimum health profiles and also the importance of requesting reference substances. She compared the agents in the rat serology screens among systems devised by COLASA, Microbiological Associates, and the ICLAS-Asian laboratories in Japan.

In the next session, Dr. Smith talked about emerging and reemerging viruses of laboratory rats and mice, including mouse and rat parvoviruses. She also highlighted mouse hepatitis virus, which has existed a long time but continues to recur and has a high prevalence in many mouse colonies including several I have encountered. The effects of many of these agents are extremely important, not only in causing overt disease but also in modifying the immunologic responses. Of course, their effects on genetically modified animals can be expected to be extremely variable as well.

Dr. Morse addressed the topic of emerging infections. Using the example of hantaviruses, he pointed out the need to avoid complacency, the need for adequate detection and diagnosis, and the importance of recognizing the role of biodiversity. There are indeed zoonoses that still remain to be identified, and animal models will be essential for studying such infectious disease. He recommended that the group take advantage of information DARPA can provide.

Dr. Goto discussed *Helicobacter hepaticus* detection and elimination using polymerase chain reaction. He also pointed out the most common types of helicobacters, which are important in causing clinical disease in mice in Japan.

Dr. Itoh talked about *H. hepaticus* as a contaminant of tumor tissues that have been passed in mice and also pointed out how to select tests for new infections. He proposed a five-tiered categorization of agents based on pathogenicity, effects on experimental results, convenience of testing, prevalence, and induction of infection. He made a plea for a testing scheme that is not overly extensive.

This very briefly summarizes the first nine presentations and related discussions.

Concluding Comments

John Vandenbergh
North Carolina State University
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I would like to share a number of recommendations that I heard without associating them with particular speakers. Many of the speakers presented very specific recommendations, which in some cases were new to me and probably were new to others.

One important discussion centered on the maintenance of genetic diversity in outbred stocks, avoiding drift and the various bottlenecks that can appear. We used terms such as *rotation*, or migration among colonies that are scattered about, and the technique of making *crosses* (such as four-way crosses).

We heard that colleagues in toxicology have not been taking advantage of information resulting from genetic analysis. I agree with that premise and refer to a recently published paper (Spearow and others 1999) showing that the strain of mice most frequently used by toxicologists, the CD1 strain, is also the least responsive to estrogen among all strains that have been tested. Yet, so much of environmental estrogen is now being tested on that particular strain of mouse. I believe it is necessary to reexamine that testing. One other suggestion that I believe requires additional thought is that of developing one or more global strains.

We learned that we can use the team approach to phenotyping. In addition, I think we can use simple but elegant behavioral assays that are available and have been described in some detail. It may be advisable to use genetically altered mice or rats as strains that can be used over a long time, rather than use only the selected strains we have. Finally, near the end of our discussion, the fascinating term *dramatype* was explained.

In closing and on behalf of the International Committee of the Council of the Institute for Laboratory Animal Research (ILAR), I want to thank all the speakers and Ralph Dell and the ILAR staff for hosting an excellent meeting. The Na-

tional Center for Research Resources of the National Institutes of Health has been very generous in providing some support for this program in addition to the Central Institute in Japan and the Japanese government. Finally, I thank Dr. Nomura who, with Dr. Held, started all of these discussions in the 1980s and who has been the driving force behind this meeting as well as the subject of normalization of animal use. Their good start and continued pressure in the area has made possible the success of this meeting.

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Appendix A

U.S./Japan Meeting
November 15, 1999
Agenda

8:45 – 9:00 a.m.	Opening Remarks Judith L. Vaitukaitis Shin-Ichi Ota
9:00 – 10:30	**Microbiologic Testing of Laboratory Mice and Rats: Uniformity of Results** *US Speakers:* Anton M. Allen, *Chair* Lela K. Riley William Shek *Japanese Speakers:* Toshio Itoh Toshiyuki Shibahara
10:30 – 10:45	Break
10:45 – 12:15 p.m.	**Emerging Microorganisms in Laboratory Mice and Rats** *US Speakers:* James Fox, *Chair* Abigail L. Smith Stephen Morse *Japanese Speakers:* Toshio Itoh Kazuo Goto
12:15 – 1:15	Lunch

1:15 – 2:45	**Genetic Evaluation of Outbred Rats** *US Speakers:* Joseph J. DeGeorge William J. White Howard Jacob *Japanese Speakers:* Tatsuji Nomura, Chair Naoko Kagiyama Hideki Katoh *UK Speaker:* Michael F.W. Festing
2:45 – 3:00	Break
3:00 – 4:30	**Defining Phenotype in Genetically Engineered Mice** *US Speakers:* Dabney K. Johnson Eugenia Floyd Jacqueline N. Crawley *Japanese Speakers:* Norikazu Tamaoki, Chair Kazunori Nakajima Tatsuji Nomura Kazuo Moriwaki
4:30 – 5:00	**Concluding Comments** John Strandberg John Vandenbergh
5:00 – 6:00	Cocktails—Executive Dining Room
6:00	Dinner—Executive Dining Room

Appendix B

Meeting Participants

Anton M. Allen, PhD, retired from National Institutes of Health
Jacqueline N. Crawley, PhD, National Institutes of Mental Health, Bethesda, MD
Joseph J. DeGeorge, PhD, Associate Director for Pharmacology and Toxicology, Center for Drug Evaluation and Research, Food and Drug Administration, Rockville, MD
Michael F.W. Festing, PhD, CStat, MRC Toxicology Unit, University of Leicester, UK
Eugenia Floyd, DVM, Pfizer Central Research, Groton, CT
James Fox, DVM, Division of Comparative Medicine, Massachusetts Institute of Technology, Cambridge, MA
Toshio Itoh, DVM, PhD, Deputy Director of ICLAS Monitoring Center, Central Institute for Experimental Animals, Kawasaki, Japan
Howard Jacob, PhD, Department of Physiology, Medical College of Wisconsin, Milwaukee, WI
Dabney K. Johnson, PhD, Oak Ridge National Laboratory, Oak Ridge, TN
Naoko Kagiyama, DVM, PhD, Head of Laboratory Animal Services, Preclinical Department, Novartis Pharma K.K., Tsukuba Research Institute, Tsukuba, Japan
Hideki Katoh, PhD, Associate Professor, Laboratory Animal Research Center, Hamamatsu University, Hamamatsu, Japan
Kazuo Moriwaki, PhD, Vice President, The Graduate University for Advanced Sciences, Kanagawa-kenn, Japan

Stephen Morse, PhD, Defense Sciences Office, Defense Advance Research Projects Agency, U.S. Department of Defense, Arlington, VA

Kazunori Nakajima, MD, PhD, Department Head, Assistant Professor, Department of Molecular Neurobiology, Institute of DNA Medicine, Jikei University School of Medicine, Tokyo, Japan.
Precursory Research for Embryonic Science and Technology (PRESTO), Japan Science and Technology Corporation (JST)

Tatsuji Nomura, MD, PhD, Director, Central Institute for Experimental Animals, Kawasaki, Japan

Shin-Ichi Ota, MA, Sci, Director of Science Information, Division of Science and International Affairs Bureau, Ministry of Education, Science, Sports, and Culture, Tokyo, Japan

Lela K. Riley, PhD, Associate Professor, Veterinary Pathobiology, University of Missouri, Columbia, MO

William Shek, DVM, PhD, Director of Diagnostics, Charles River Laboratories, Wilmington, MA

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John Strandberg, DVM, PhD, Director, Comparative Medicine, National Center for Research Resources, National Institutes of Health, Bethesda, MD

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John Vandenbergh, PhD, Professor, Department of Zoology, North Carolina State University, Raleigh, NC