Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

PART II

Murine Models

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1 Management of Immunocompromised and Infected Animals

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CONTENTS

INTRODUCTION

The field of immunology has increasingly developed over the last decades and now requires a wide range of methods. Beginning with the application of attenuated infectious agents for vaccine production in integer animals, the majority of experiments today are performed *in vitro*, (e.g. phagocytosis, cytotoxic activity, signal transduction). However, to cover the complexity of the immune system, additional *in vivo* experiments are indispensable.

In the last decade naturally occurring and artificially induced immunodeficient animals have been widely used to study different aspects of immunity (Viney, 1994), such as autoimmunity (Benoist and Mathis, 1993), T-cell receptor repertoire (Mittruecker *et al.*, 1995), B cell compartment (Gu *et al.*, 1991), effects of adhesins (Mayadas *et al.*, 1993), functions of cytokines (Kopf *et al.*, 1995a,b; Trinchieri, 1997) and pathogenesis of

METHODS IN MICROBIOLOGY, VOLUME 25
ISBN 0-12-521528-2

infections (Kaufmann and Ladel, 1994). From these studies, the high complexity and considerable redundancy of the immune system became evident. In the context of managing immunodeficient animals, the finding of a spontaneously developing chronic ulcerative enterocolitis (inflammatory bowel disease) in a T-cell receptor mutant (Mombaerts *et al.*, 1993), Il-2 (Sadlack *et al.*, 1993; Mähler *et al.*, 1996) and Il-10 deficient mice (Kühn *et al.*, 1993) is of interest.

A large number of the transgenic mutants used in particular in the field of immunology are immunodeficient, being more or less susceptible to infections. Therefore, optimal hygienic standards are indispensable for these animals. We will try to point out here the special requirements for the management, breeding and housing of immunocompromised and infected animals, especially those for the mouse and rat.

◆◆◆◆◆◆ MICROBIOLOGICAL STANDARDIZATION

The quality of laboratory animals, mainly rodents, has improved during the last decade. The first attempts at eliminating disease were made in the 1950s. At that time infectious agents were widespread in rodent colonies, and many experiments were interrupted by infections. It became obvious that classical veterinary approaches, such as improved husbandry, vaccination, antibiotics and chemotherapeutics, would not eliminate pathogens, and therefore gnotobiotic techniques such as caesarean derivation and subsequent raising in isolation were established. This resulted in the elimination of various organisms, such as *Mycoplasma pulmonis*, which had previously been ineradicable. However, infections were still prevalent in many colonies. More sophisticated experimental procedures were increasingly sensitive to the influence of viruses. Some viruses had been tolerated in the past as they have a low potential to induce clinical disease, but both scientists and breeders were aware of their presence. It was shown later that many of these agents, although clinically silent, can induce increased variation between individuals and can influence biochemical or immunological functions. Research complications occurred frequently, resulting in the need to eliminate also those agents that cause clinically silent infections, and to monitor colonies of rodents for the presence or absence of such organisms.

Today, it is generally accepted that good research requires animals that are free from micro-organisms that might influence the health of the animals (or humans) or the results of experiments.

Influence of Micro-organisms on Research Results

It is generally accepted that research complications due to overt infectious diseases are significant and that clinically ill animals should not be used for scientific experiments. The effect of clinically silent infections, however, may be devastating, because they often remain undetected. Scientists in general are not well informed of such influences on their research. Only a small percentage of detected complications has been

published. The literature is scattered across diverse scientific journals, and many articles are difficult to locate. To address this problem, conferences have been held on viral complications on research, and the knowledge available summarized in conference proceedings (Bhatt *et al.*, 1986a; Hamm, 1986). The problem has been reviewed by Lussier (1988), the National Research Council (1991) and Hansen (1994).

Research complications may occur in various ways. Although acute clinical signs may not be observed, infected animals may show altered behaviour, suppressed body weight, or reduced life-expectancy, which may, for example, influence the tumour rate. Micro-organisms present in an animal may lead to contamination of samples and tissue specimens such as cells, tumours, sera and monoclonal antibodies. This may interfere with experiments performed with cells or isolated organs.

The experiment itself may be a stress factor and increase the sensitivity to an agent, and thus induce clinical disease or death. Environmental factors, such as increased temperature or relative humidity (for example, in metabolic cages), may induce stress which activates latent infections resulting in lung complications caused by *Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus aureus* or *Pneumocystis carinii,* especially in immunodeficient animals. Naturally, various micro-organisms can interact and lead to clinical disease or research complications, which are dependent on the combination of micro-organisms.

The disease rate is not only dependent on the host, but also on specific properties of the infectious agents. There are different strains of many viruses, with different organotropism (e.g. hepatotropic, enterotropic and neurotropic strains of the mouse hepatitis virus (MHV)). This influences the disease rate and the mortality, as well as the type and severity of pathological changes. For example, the immunosuppressive variant of the minute virus of mice (MVMi) replicates in lymphocytes, whereas the prototype strain (MVMp) replicates in fibroblasts, thus resulting in different effects on animals or experiments. Both variants usually do not induce clinical disease, but may affect various parameters such as wound healing, immunological reactivity, tumour growth and development, embryonic development and birth rate.

Various effects are possible on the function or the morphology of organs or cell systems. Histopathological changes that resemble adenomas have been observed in the trachea or bronchioles during the regenerative phase after a Sendai virus infection.

When pathogens infect laboratory animals, the immune system is activated regardless of the level of pathogenicity. Many micro-organisms have the potential to induce functional suppression or stimulation of the immune system. Sometimes, only T cells, B cells or specific subpopulations are influenced. Therefore, most virus infections and infections with bacteria or parasites are detrimental to immunological research and must be avoided.

Some micro-organisms have a specific effect on enzymatic or haematological parameters. Lactate dehydrogenase virus (LDV) can induce an up to 100-fold increase in the activity of lactate dehydrogenase (LDH) and other enzymes in the plasma. Numerous reports exist in the literature about

modulation of oncogenesis. Infectious agents may induce cancer, enhance chemical or viral carcinogenesis, or reduce the incidence of cancer. Some organisms even influence the growth rate of transplantable tumours.

Immunosuppressed animals are usually more sensitive to infections than are immunocompetent animals. Infections in immunodeficient animals frequently result in increased mortality due to a reduced or absent resistance to low pathogenic or even commensal micro-organisms.

It is important for various reasons that animals used for infection studies are free from adventitious infections. The infection in question might be influenced by an adventitious organism by means of immunomodulation and, therefore, result in increased or reduced resistance to experimental

Table 1. Mouse hepatitis virus (MHV): examples of interference with research

Effect of virus	Reference
Immunology	
Virus replication in macrophages, macrophage dysfunction	Boormann *et al.* (1982)
Dysfunction of T and B cells	de Souza *et al.* (1991)
Activation of NK cells, alteration of immune responsiveness	Schindler *et al.* (1982)
Immunosuppression or immunostimulation, depending on the time of infection	Virelizier *et al.* (1976)
Reduced levels of cytokines, γ-interferon and cytokines in spleen cells	de Souza *et al.* (1991)
Permanent decrease in skin graft rejection and T-cell-dependent antibody responses after recovery from infection	Cray *et al.* (1993)
Microbiology	
Reduced susceptibility to viral infections (Sendai, PVM)	Carrano *et al.* (1984)
Enhanced resistance to *Salmonella* infections	Fallon *et al.* (1991)
Confusion about the origin of Tettnang virus isolates	Smith *et al.* (1983)
Physiology	
Alteration of liver enzyme levels	Barthold (1986)
Altered protein synthesis	Lucchiari *et al.* (1992)
Changes in peripheral blood	Piazza *et al.* (1965)
Increased monocyte procoagulant activity	Levy *et al.* (1981)
Decrease in the incidence of diabetes in non-obese diabetic mice	Wilberz *et al.* (1991)
Oncology	
Abnormal tumour passage intervals or tumour invasion pattern	Manaker *et al.* (1961)
Rejection of human xenografts in the nude mouse	Kyriazis *et al.* (1979)
Contamination of transplantable tumours	Nicklas *et al.* (1993a)

NK, natural killer; PVM, pneumonia virus of mice.

infection. Micro-organisms resulting from a natural infection might contaminate viruses, bacteria or parasites that are passaged in laboratory animals. Spontaneous infections may lead to false conclusions. For example, the first isolations of Sendai virus were made from mice that had been inoculated with diagnostic materials from humans and swine. In subsequent years, evidence accumulated to show that an indigenous virus of mice had been isolated (National Research Council, 1991).

Some examples of virus interference with research are given in Table 1 for a mouse virus (MHV) and in Table 2 for a rat virus (Kilham rat virus (KRV)).

Table 2. Kilham rat virus (KRV): examples of interference with research

Effect of virus	Reference
Immunology	
Infection of T and B lymphocytes and suppression of various lymphocyte functions	McKisic *et al.* (1995)
Stimulation of autoreactive T lymphocytes specific for pancreatic antigens	Brown *et al.* (1993)
Altered susceptibility to autoimmune diabetes in rats	Guberski *et al.* (1991), Ellermann *et al.* (1996)
Altered cytotoxic lymphocyte activity	Darrigrand *et al.* (1984)
Depression of lymphocyte viability and various T-cell functions	Campbell *et al.* (1977a,b)
Stimulation of interferon production	Kilham *et al.* (1968)
Microbiology	
Supports secondary colonization with other micro-organisms	Carthew and Gannon (1981)
Influence on the prevalence of *Yersinia*-induced arthritis in rats	Gripenberg-Lerche and Toivanen (1993, 1994)
Persistent infection of cell lines	Wozniak and Hetrick (1969)
Physiology	
Inhibition of lipid formation in rat kidney cells *in vitro*	Schuster *et al.* (1991)
Increased leukocyte adhesion in the aortic epithelium	Gabaldon *et al.* (1992)
Congenital malformation	Margolis and Kilham (1975)
Death and resorption of fetuses	Kilham and Margolis (1966)
Oncology	
Suppression of leukaemia induction by Moloney virus	Bergs (1969)
Containment of leukaemias or leukaemia virus preparations	Spencer (1967)
Contamination of tumours	Campbell *et al.* (1977b)

Principles of Health Monitoring

The microbiological quality of laboratory animals is a direct result of colony management practices, and monitoring provides an after-the-fact assessment of the adequacy of those practices. Monitoring is, therefore, of greatest value in connection with maintenance of animals in isolation systems where vigorous microbiological control is applied.

Health monitoring procedures in animal populations differ from the procedures used in human medicine. Especially in populations of small laboratory animals, such as mice and rats, a single animal has only a limited value. Health monitoring of laboratory rodents aims at detecting health problems or defining the pathogen status in a population rather than in an individual. Therefore, systematic laboratory investigations (health surveillance programmes) are necessary to determine the colony status and, most importantly, to prevent influences on experiments. Disease diagnosis differs from monitoring in that abnormalities are the subject of testing. This testing is not scheduled, and tests are directed towards identifying those pathogens most likely to cause the lesion.

Routine monitoring programmes will primarily focus on infectious agents. Most infections are subclinical, but can nevertheless modify research results. Therefore, detection of the presence of infectious agents, whether or not they cause clinical disease, is necessary. Monitoring must include animals in the colony and all relevant vectors by which micro-organisms may be introduced into a colony. Therefore, it may be necessary, particularly in experimental units, that monitoring is not restricted to animals, and that other materials that pose a risk (e.g. biological materials) be monitored to prevent the introduction of agents into a facility.

The need for health surveillance programmes is generally accepted, but there is a great diversity of opinion about their design. Every institution requires an individual programme that has to be tailored to the conditions it is to serve. Most importantly, although the programme is dependent on research objectives, numerous additional factors must be considered, such as the physical conditions and layout of the animal house, husbandry methods and sources of animals. The type of programme further is influenced by the number as well as the quality of personnel, and by finances. It may even be necessary in a multipurpose unit to have a range of different programmes (e.g. one for isolator-housed and one for barrier-housed animals).

There is always a risk that infectious agents might be introduced, especially into experimental units. This risk has to be taken into consideration when the monitoring programme is designed. More frequent monitoring is reasonable if the risk of introducing unwanted organisms is high (e.g. if animals or biological materials are frequently introduced or if many personnel need access to the animals). Simulation experiments have shown that small and frequent samples are more suitable for detecting an infection than larger samples taken at less frequent intervals (Kunstyr, 1992).

Various designs of monitoring programmes have been published or presented on scientific meetings. General aspects of health surveillance

are provided by the Committee on Infectious Diseases of Mice and Rats (National Research Council, 1991). Recommendations exist about how monitoring of breeding colonies (Kraft *et al.*, 1994) or experimental colonies (Rehbinder *et al.*, 1996) should be conducted. An overview of the monitoring of experimental rodent colonies has been given by Nicklas (1996).

Animals

In general, the animals are the most crucial point in a monitoring programme. Their status has to be defined, and they are the most important source of infection. Proper sampling is therefore necessary in order to detect an infection in a given population as early as possible. Animals coming from outside have to be checked to assess or exclude the risk of introducing unwanted organisms, and animals already within the unit are monitored to define their status and to obtain information on the presence or absence of infectious agents in the colony. It is obvious that a sufficient number of animals has to be monitored. Based on a recommendation by the ILAR Committee on Long-Term Holding of Laboratory Rodents (1976), it has become common practice to monitor at least eight randomly sampled animals, which is (theoretically) sufficient to detect an infection with a 95% probability if at least 30% of a population is infected. Monitoring animals of different ages is useful, because younger animals often have a greater parasite or bacterial burden, whereas older animals ($\geq$3 months) are more suitable for detecting viral infections.

Sentinels/'control' animals

Random sampling for monitoring is not a serious problem in breeding colonies, but it is usually impossible in experimental units or not reasonable in the case of immunodeficient animals. Immunodeficient animals may not be able to produce sufficient amounts of antibodies, and so their status can be evaluated only by the use of sentinels. It is therefore advisable to have sentinel animals in each experimental unit in order to evaluate the status of a population. Such animals should be kept in such a way that they receive maximum exposure to potential infections. If sentinels are not bred within the colony that is being monitored, they must be obtained from a breeding colony of known microbiological status, i.e. they must be negative for all rodent pathogens. The sentinel animals must be housed for a sufficiently long time in the population that is to be monitored in order to develop detectable antibody titres (for serology) or parasitic stages. It is common to house sentinels in a population for at least 4–6 weeks prior to testing, longer periods are even better. In most cases, outbred animals are used as sentinels, because they are cheaper and more resistant to clinical disease than are inbred animals. Inbred animals may in specific cases (e.g. for virus isolation) be more valuable as sentinels, because they may be more sensitive to an agent and thus more likely to develop clinical disease. In other cases, their extreme

or even complete resistance to specific agents may be a reason to use specific strains with known characteristics. For example, C57BL/6 or DBA/2 mice are sensitive to clinical infections with MHV, whereas A/J mice are resistant to this virus. On the other hand, C57BL/6 mice are resistant to ectromelia virus (Bhatt and Jacoby, 1987). This virus causes high mortality with typical skin lesions in C3H mice, and high mortality but minimal skin lesions in CBA and DBA/2 mice. Use of immunodeficient animals, such as thymus-aplastic nude mice, as sentinels may increase the sensitivity if specific bacterial pathogens such as *Pasteurella pneumotropica*, parasites (e.g. *Spironucleus muris*) or viruses are to be detected in a population. In the past, injection of cortisone to suppress the immune system was recommended. Cortisone results in overgrowth and thus makes it easier to detect bacterial pathogens directly. However, cortisone tests have lost importance as the direct demonstration of microorganisms can now be performed more easily by means of molecular methods such as the polymerase chain reaction (PCR).

A multitude of physiological characteristics can be influenced by introducing a transgene into the genome or by gene targeting. Changes of the immune status frequently arise, resulting in immune defects or immunosuppression. As a consequence, there may not only be altered sensitivity to pathogenic agents, but also suppression or lack of antibody response. When monitoring an immunodeficient colony, to avoid false-negative results in serological tests animals whose immune responsiveness is well known (e.g. old vasectomized males, retired breeder females) should be used as sentinels in order to obtain reliable serological results. It is advisable for classical barrier systems to have sentinel animals in each animal room. The animals should be housed in various locations on the bottom shelves, without filter tops. Each time the cages are changed, soiled bedding from different cages should be transferred to sentinel cages.

During the last decade additional housing systems such as microisolators, individually ventilated cages and filter cabinets (see pages 134–135) have emerged. These offer the advantage of separating small populations from each other and are frequently used for housing immunodeficient, immunosuppressed or infected animals, because they very efficiently prevent transmission of infectious agents. Each isolator or microisolator cage must therefore be considered as a self-contained microbiological entity. Health monitoring under such housing conditions as well as monitoring isolator-housed animals can only be conducted by the use of sentinel animals. Due to limited space, less than the recommended number of animals are available in many cases, which is acceptable if sentinels are properly housed. In the case of isolators, a realistic number of sentinel animals is housed in one or several cages (depending on the isolator size) on soiled bedding taken from as many cages as possible. In most cases, only 3–5 animals per isolator will be available for monitoring.

If animals are housed in microisolators or in individually ventilated cages, sentinels must be housed in filter-top units like other animals. When cages are changed in changing cabinets, soiled bedding from several cages is transferred into a separate cage which is used to house

sentinels. Weekly changes of donor cages will give a representative insight into the microbiological status of the whole population.

Frequency of monitoring

The frequency of monitoring will depend on various factors, but mainly on the importance of a pathogen to the use of the population and on the level of risk of infection for a population. Naturally, economic considerations are important as well. Both of the recommendations of the Federation of European Laboratory Animal Science Associations (FELASA) (Kraft *et al.*, 1994; Rehbinder *et al.*, 1996) state that monitoring should be conducted quarterly. Most commercial breeders of laboratory rodents monitor more frequently (every 4–6 weeks). In most multipurpose units housing immunodeficient or infected animals, more frequent monitoring is preferable as this will result in earlier detection of an infection. As a general rule, it is advisable to monitor a small number (e.g. 3–5) of animals from each unit every 4–6 weeks instead of 10 animals every 3 months. Under practical conditions, not every animal may be monitored for all micro-organisms. Depending on the factors already mentioned, the frequency of testing may be different for different agents. Monitoring for more frequently occurring organisms or for zoonotic or otherwise important agents will be performed more frequently (monthly), whereas testing for unusual organisms like K-virus or polyoma virus can be done less frequently (e.g. biannually or annually). Results obtained from monitoring of sentinels are valid for all animals of the same species within a population, irrespective of the experiment or animal strain. Independent from animals which are scheduled for monitoring, all animals with clinical disease should be submitted for direct examination for micro-organisms (bacteria, parasites, viruses) and for histopathology.

Biological materials

In addition to animals, other materials may carry unwanted micro-organisms and may be important sources of infection. Immunodeficient nude mice are often used for tumour transplantation studies and are at risk of infections transmitted via the transplanted tissue. In many cases, organisms have been introduced into animal populations by contaminated tumours or leukaemias (Collins and Parker, 1972; Nicklas *et al.*, 1993a). Monoclonal antibodies (Nicklas *et al.*, 1988) and virus suspensions (Smith *et al.*, 1983) used for infection studies might also be contaminated, and these must be monitored before use in animals.

Agents

A decision has to be made in each facility about which organisms are acceptable or unacceptable. Lists of infectious agents to be monitored in routine programmes have been published by various organizations (Kunstyr, 1988a; National Research Council, 1991; Kraft *et al.*, 1994; Waggie *et al.*, 1994) and can be used for guidance. Monitoring for all the

agents mentioned (mycoplasmas, bacteria, bartonellas, fungi, spirochaetes, protozoans, helminths, arthropods) on a routine basis is neither realistic nor necessary. The most important micro-organisms are those that are indigenous and pose a threat to the research or to the health of the animals and humans and, in addition, those which can be eliminated. Therefore, oncogenic retroviruses are excluded as they integrate into the mammalian genome, and thus cannot be eradicated by presently available methods. Other micro-organisms may be less important as they are unlikely to occur in good quality rodents due to repeated rederivation procedures (e.g. *Brucella, Erysipelothrix*). Most cestodes are unlikely to be found, since they require an intermediate host. In the case of immunocompromised animals or in infection experiments, however, monitoring for a comprehensive list of micro-organisms (some examples are given in Box 1) is reasonable. Various micro-organisms that usually do not cause clinical signs in immunocompetent animals (e.g. *Staph. aureus, Pseud. aeruginosa, Pneum. carinii*) may cause serious problems in immunodeficient animals. It is

Box 1 Examples of bacterial and fungal pathogens and parasites that should not be detectable in barrier-housed colonies of mice and rats

Bacteria
Actinobacillus muris
Actinobacillus sp.
Bordetella bronchiseptica
CAR bacillus
Citrobacter rodentium
Clostridium piliforme
Corynebacterium kutscheri
Erysipelothrix rhusiopathiae
Haemophilus sp.
Haemophilus influenzaemurium
Helicobacter sp.
Klebsiella pneumoniae
Klebsiella oxytoca
Listeria monocytogenes/ivanovii
Pasteurella multocida
Pasteurella pneumotropica
Other Pasteurellaceae
Pseudomonas aeruginosa
Salmonella sp.
Staphylococcus aureus
Streptobacillus moniliformis
Streptococcus pneumoniae
β-Haemolytic Streptococci
Yersinia pseudotuberculosis

Mycoplasmas
Mycoplasma pulmonis
Mycoplasma arthritidis
Mycoplasma neurolyticum

Fungi
Trichophyton sp.
Microsporum sp.
Yeasts

Parasites (all parasites)
Aspiculuris tetraptera
Syphacia obvelata
Syphacia muris
Trichosomoides crassicauda
Hymenolepis sp.
Spironucleus muris
Coccidia
Giardia sp.
Trichomonads
Amoebae
Demodex sp.
Myobia musculi
Myocoptes musculinus
Notoedres sp.
Polyplax spinulosa
Radfordia affinis
Radfordia ensifera

therefore necessary that immunodeficient animals are monitored not only for strong or weakly pathogenic organisms, but also for opportunistic pathogens or commensals. Micro-organisms with a low pathogenic potential can cause clinical signs of disease if animals are infected with several agents (e.g. KRV and *Past. pneumotropica* (Carthew and Gannon, 1981)). In other cases, different micro-organisms of low clinical importance may interact and have a severe impact on research results such as oncogenic viral expression (Riley, 1966).

Each institution should prepare a list of those organisms that are not acceptable in the colony or in parts of it. The list is easiest to establish for viruses (for an example, see Table 3). A large amount of information is available on their pathogenic potential and on their ability to compromise the object of research. Monitoring for viruses can be done selectively by serological methods. Only a few exceptions exist, e.g. parvoviruses that cross-react in indirect immunofluorescence or enzyme-linked

Table 3. Serologic tests for the detection of infectious agents in mice and rats

Infectious agent	Recommended methods	Species
Viruses		
Mouse hepatitis virus (MHV)	ELISA, IIF	Mouse
Rat corona viruses (RCV/SDAV)	ELISA, IIF	Rat
Kilham rat virus (KRV)	HI, ELISA, IIF	Rat
Toolan's H-1 virus	HI, ELISA, IIF	Rat
Minute virus of mice (MVM)	HI, ELISA, IIF	Mouse
Pneumonia virus of mice (PVM)	ELISA, IIF, HI	Mouse, rat
Reo virus type 3	ELISA, IIF	Mouse, rat
Sendai virus	ELISA, IIF, HI	Mouse, rat
Mouse encephalomyelitis virus (GD VII)	ELISA, IIF, HI	Mouse, rat
Mouse adenovirus (FL, K87)	ELISA, IIF	Mouse, rat
K-virus	HI	Mouse
Polyoma virus	ELISA, IIF, HI	Mouse
Lymphocytic choriomeningitis virus (LCMV)	ELISA, IIF	Mouse
Ectromelia virus	ELISA, IIF	Mouse
Hantaviruses	ELISA, IIF	Rat
Mouse rotavirus (EDIM)	ELISA, IIF	Mouse
Lactic dehydrogenase elevating virus (LDV)	PCR, enzyme test	Mouse
Bacteria		
Mycoplasma pulmonis	ELISA, IIF, culture	Mouse, rat
Mycoplasma arthritidis	ELISA, IIF, culture	Rat
Clostridium piliforme	IIF	Mouse, rat
CAR bacillus	ELISA	Rat

ELISA, enzyme-linked immunosorbent assay; HI, haemagglutination inhibition assay; IIF, indirect immunofluorescence assay; PCR, polymerase chain reaction.

immunosorbent assay (ELISA) tests (Jacoby *et al.*, 1996) and sometimes cannot be identified unequivocally. For some viruses (e.g. K virus, polyoma virus) the only question is whether or not monitoring is necessary, because they have been eradicated from the vast majority of rodent colonies many years ago. Only few new rodent viruses have been detected during the last few years, e.g. mouse parvovirus (MPV) and rat parvovirus (RPV) (Jacoby *et al.*, 1996), and it has to be expected that new rodent viruses will be isolated, although only occasionally.

Less is known about the ability of most parasites to influence research results. They are considered to be a hygiene problem and are therefore eradicated from rodent colonies. Some protozoans, such as trichomonads, are occasionally detectable in pathogen-free animals from commercial breeders. They are considered to be apathogenic, and nothing is known about their influence on the physiology of animals. They are, however, likely to be species specific, and thus might be an indicator of a leak in the system or of the existence of direct or indirect contact with wild rodents. The most complex problems exist for bacteria. In contrast to viruses their importance in laboratory animals is usually estimated on the basis of their ability to induce pathological changes or clinical disease, since almost nothing is known about most rodent bacterial species with regard to their potential to cause other effects on their hosts and on experiments. Insufficient information exists on the taxonomy and proper identification for various rodent-specific bacterial species such as *Past. pneumotropica* or other members of the Pasteurellaceae (e.g. *Haemophilus influenzaemurium, Actinobacillus muris*). Lack of detailed information on the characteristics of these organisms together with the presently unclear taxonomic situation often leads to misidentification, and the lack of knowledge about species specificity impedes their elimination. The FELASA working group on animal health (Rehbinder *et al.*, 1996) therefore decided to recommend that rodents should be monitored for all Pasteurellaceae. There is, however, evidence, that some growth-factor-dependent Pasteurellaceae found in rodents are closely related to *Haemophilus parainfluenzae* and might therefore be transmitted by humans (Nicklas *et al.*, 1993b). It is unclear if these bacteria can be eradicated permanently from barrier units, because exposure of barrier-produced animals to humans represents a permanent risk for reinfection. The same is true for several members of the Enterobacteriaceae (*E. coli, Klebsiella, Proteus), Staph. aureus* and *Pseud. aeruginosa*, for which humans are the reservoir. Another problem arises from the fact that many bacteria are presently being reclassified, resulting in changes in their names. For example, the mouse-specific organism known as *'Citrobacter freundii* 4280' has recently been reclassified as *Citrobacter rodentium* (Schauer *et al.*, 1995). Whole genera have been renamed, and additional bacterial species have been detected, e.g. *Helicobacter hepaticus, Heli. muridarum* and *Heli. bilis* (Lee *et al.*, 1992; Fox *et al.*, 1994, 1995). Some of these fastidious organisms are not detected or not properly identified by all monitoring laboratories. Adding such known pathogens to a list for which animals should be monitored may be unrealistic as long as proper methods for their detection and identification are not readily available in a monitoring laboratory.

A list of pathogens should contain all indigenous micro-organisms for which rodents are the infectious reservoirs and other micro-organisms that might be of importance for the research conducted with such animals. The list of these additional organisms may be long in the case of immunodeficient animals. The whole spectrum of micro-organisms as a concept is not a permanent list for all time, it rather represents a moving boundary in which old pathogens are eradicated and new pathogens are added. In practice, such lists of agents do not differ much between different facilities or commercial breeders. Monitoring for micro-organisms is usually done by commercial laboratories, and is thus determined by their capabilities (some of the larger research institutes have dedicated diagnostic laboratories). It is important that all investigations should be performed in laboratories with sufficient expertise in microbiology or pathology of the relevant species. Serological tests also require technical competence to ensure sufficient standardization of tests (including controls) and accurate interpretation of results.

Testing of animals usually starts with necropsy and blood sampling for serology, followed by microscopic examination for parasites and sampling of organs for bacteriology, pathology and, in rare cases, virological examinations. For financial reasons, bacterial culture is often restricted to very few organs. Monitoring more organs would, however, increase the probability of detecting bacterial pathogens in an animal. Bacterial cultures should be done for the respiratory tract (nasal cavity, trachea, lungs), intestinal tract (small and large intestine) and urogenital tract (vagina, prepuce, uterus, kidney). In the case of pathological changes, additional organs (liver, spleen, mammary gland, lymph nodes, conjunctiva, etc.) should be cultured.

Serology is easy and cheap to perform, and serum samples can be mailed easily. Whole-body examinations including bacteriology and parasitology are more expensive, and live animals must be shipped to the monitoring laboratory. Therefore, many laboratories monitor only serologically. Meanwhile, serological methods exist to detect some bacterial infections, but these are not generally accepted, and only a few laboratories apply these methods. At present, the method of choice for the detection of most bacterial pathogens is bacterial culture, and thus should be part of every monitoring programme.

Sources of Infection

Keeping rodents free of pathogens in research facilities is a much more complex problem than in breeding colonies. Animals and various experimental materials need to be introduced into experimental facilities. In addition, more personnel must have access to animals due to the requirements of the experiments. This results in a higher risk of introducing pathogens.

Effective measures must be taken to standardize laboratory animals microbiologically as far as possible. Therefore, the design of modern laboratory animal buildings is based mainly on microbiological concepts aimed at the prevention of infections. These measures are responsible for

a high percentage of the expense arising from planning and constructing an animal house. Furthermore, high running costs are taken into account for energy, hygienic precautions, and personnel to avoid infections during operation.

In addition to constructive measures, an appropriate management system is necessary for the prevention of infections, as well as for their detection and control. It is a major task for the management of an animal facility to understand how micro-organisms might be introduced or spread under the specific conditions given. Management of all animal facilities in an institution is best centralized. This warrants that all information dealing with the purchase of animals, use of experimental materials and equipment, as well as the performance of animal experiments flows through one office. This reduces the opportunity for failures of communication. Centralized management can best establish comprehensive monitoring programmes to evaluate important risk factors such as animals and biological materials before they are introduced into a facility. Contamination of animals can happen in two ways. One has to distinguish between the introduction of micro-organisms coming from outside and the transmission of micro-organisms within a colony. Both can be influenced by the management and the housing system.

Animals

The greatest risk of contamination of any animal arises from another animal of the same species. Most facilities are multipurpose, and must therefore house a variety of strains coming from various breeding units. In addition, many specific strains or transgenic animals are available only from research institutes. Still, animals are the most important risk factor, even if their quality has constantly improved during the last decades.

As a general rule, all animals coming from sources of unknown microbiological status should be regarded as infected unless their status has been defined. This is especially important when transgenic animals are introduced from other experimental colonies. These animals must be housed separately from others. The risk of introducing pathogens via animals from external sources is lower when animals are available from very few sources of well-known microbiological status and if these animals have been protected from contamination during shipment. In many cases direct transfer of such animals without quarantine into an experimental unit will be necessary; however, spot checks should be performed from time to time to redefine the status upon arrival. In many cases it is acceptable to introduce animals from microbiologically well-known (external) colonies into experimental units, but never into a breeding unit, especially if many different strains and/or transgenic lines are co-maintained. In the latter case new breeders should only be introduced via embryo transfer or hysterectomy (see pages 162–163). Outbred mice or F1 hybrids are generally used as surrogate and foster dams and can easily be bred in the transgenic unit, as is the case for the sterile males required to induce pseudocyesis in the surrogate dams.

It must be emphasized that a specific risk of transmitting micro-organisms may arise from immunodeficient animals. Many virus infections (MHV, RCV/SDA, Sendai, PVM) are limited in immunocompetent animals, and the virus may be eliminated completely. Immunodeficient animals may, however, shed infectious virus for longer periods of time, or may be infected persistently.

Like animals of unknown status, animals known to be infected must always be housed in isolation. This can best be done in isolators or, if proper handling is guaranteed, in microisolator cages or in individually ventilated cages.

Biological materials

Biological materials represent a high risk if they originate from or have been propagated in animals. In particular, tumours, viruses or parasites that are serially passaged in animals often pick up pathogens, and therefore a high percentage of these are contaminated. Many murine viruses (e.g. MVM, K virus, mouse encephalomyelitis virus and mouse adenovirus) were first isolated from contaminated virus pools or (e.g. polyoma virus, Kilham rat virus (KRV), Toolan's H-1 virus) from contaminated tumours. Such materials can be stored frozen without loss of infectivity, and may be hazardous to humans or laboratory animals even after decades. The problem of viral contamination in biological materials became obvious in the studies done by Collins and Parker (1972). They monitored 475 murine leukaemias and tumours and found viral contamination in 69% of the samples. The same percentage of contaminated mouse tumour samples was found by Nicklas *et al.* (1993a) after animal passages. Many organisms disappear under *in vitro* conditions, so that the contamination rate after these passages is lower. Among the contaminants, lymphocytic choriomeningitis virus (LCMV) (Bhatt *et al.*, 1986b) and hantaviruses (Yamanishi *et al.*, 1983) have repeatedly been found, and outbreaks in humans associated with infected animals or with contaminated tumour material have been reported (Kawamata *et al.*, 1987).

Pathogenic micro-organisms can also be transmitted by other contaminated materials of animal origin, such as monoclonal antibodies (Nicklas *et al.*, 1988) and viruses (Smith *et al.*, 1983). Contamination of biological materials is not restricted to viruses. *Myc. pulmonis* and other bacterial pathogens such as *Past. pneumotropica* have been found in tumours (Nicklas, 1993). Additional pathogens (*Eperythrozoon* sp., *Haemobartonella* sp., *Encephalitozoon* sp.) can contaminate biological materials after animal-to-animal passage (National Research Council, 1991) and thus may be transmitted to recipient animals.

Humans

Humans can act as mechanical or biological carriers of micro-organisms. Humans are unlikely to be an appropriate host where murine pathogens

can reside and replicate. However, the importance of humans as mechanical vectors should not be underestimated, and several human pathogens can cause infections in rodents, at least in immunodeficient animals. It has to be assumed that each micro-organism that is present in humans who have access to a barrier unit might sooner or later colonize the animals. Transmission certainly cannot be avoided in barrier-maintained colonies, even by wearing gloves and surgical masks and taking other precautions. It may only be avoided by establishing strict barriers as provided by isolator maintenance. Immunodeficient animals, at least animals used for breeding or long-term experiments, which are known to have an increased sensitivity to infection with bacteria of human origin (*Staph. aureus, Kleb. pneumoniae, Esch. coli,* etc.) should, therefore, be housed in isolators or microisolators (individually ventilated cages).

Little published information is available on the role of humans as mechanical vectors. There is no doubt that micro-organisms can be transmitted by handling (La Regina *et al.*, 1992). Micro-organisms can even be transported from pets to laboratory animals by human vectors (Tietjen, 1992). Such examples emphasize the need for proper hygienic measures and the importance of positive motivation of staff. It is an important task of the management of an animal facility to ensure that personnel coming into contact with animals have no contact with animals of lower microbiological quality.

Vermin

Vermin are another potential source of infections. Flying insects do not present a serious problem because they can easily be removed from the incoming air by means of filters or insect-electrocuting devices. Crawling insects such as cockroaches are more difficult to control, and cannot be excluded with certainty. The most serious problem arises from wild rodents, which are frequently carriers of infections. Wild, as well as escaped, rodents are attracted by animal diets, bedding and waste. Modern animal houses usually have devices that normally prevent entry of vermin.

Possible routes of infection of laboratory animals have been discussed in more detail by Nicklas (1993).

Present Status of Laboratory Animals

Since serological testing has been possible, many laboratories have evaluated the murine viral status of rodent colonies. Managers of animal facilities had to learn techniques to prevent, control and eradicate infection and means of adapting the facilities for their own purposes. As a consequence, the diversity of viruses and the frequency with which they are detected has declined markedly. Virus infections have now been almost entirely eradicated from most commercial breeding colonies. This

gave animal care unit administrators and researchers the opportunity to procure and maintain virus-free stocks, and researchers to use better standardized animals for research. However, this progress of eradication has not occurred without periodic shut-downs at breeders' and users' facilities.

Reports on the prevalence of virus infections in rodents throughout the world have been published frequently. An overview given by the National Research Council in 1991 demonstrates that the majority of colonies were at that time infected with 3–4 viruses. It has to be expected that more recent statistics would reveal that the prevalence of murine viruses has declined further. However, most facilities house at least small numbers of infected animals or animals of unknown status. Many small or decentralized facilities do not monitor at all. Today, murine parvoviruses and MHV are the most prevalent agents in rodents. Especially for parvoviruses the situation is unclear, because recently described parvoviruses have not yet been sufficiently characterized, and only limited knowledge exists about their prevalence. Various viruses are still prevalent at a low level. These can emerge unexpectedly, as occurred a few years ago when a sudden outbreak of ectromelia was observed in the USA (Dick *et al.*, 1996). This virus had not been detected in the USA for many years.

The situation is very similar for bacterial pathogens and parasites. Most of these were eradicated when the principles of gnotobiology were introduced into laboratory animal science. A few parasites (pinworms, mites, protozoans) are still endemic in various rodent colonies, but most of the primary bacterial pathogens (Salmonellae, *Corynebacterium kutscheri, Leptospira, Streptobacillus moniliformis*) are no longer detected in well-run facilities, although they may re-emerge as shown recently (Wullenweber *et al.*, 1990; Koopman *et al.*, 1991). *Clostridium piliforme*, which is the causative agent of Tyzzer's disease, and *Myc. pulmonis*, are detected more frequently. Most experimental colonies and some commercial breeders' colonies are positive for Pasteurellaceae like *Past. pneumotropica* and *Actinobacillus muris*. The real prevalence of organisms belonging to this family is not definitely known, due to difficulties in identification. The situation is also unclear for *Helicobacter* species, because these cannot be detected in all monitoring laboratories. It has to be expected that these, too, are widespread in laboratory rodents. Such organisms have in the past been spread by animals that had become infected long before the organisms had been detected. It is, therefore, extremely important that germ-free or gnotobiotic animals, rather than SPF animals, are used for hygienic rederivation in order to avoid this problem in the future.

A number of additional disease agents such as group B and G streptococci, *Staph. aureus, Haem. parainfluenzae, Corynebacteria* spp. inducing scaly skin disease, and others have been found in so-called pathogen-free rodents during the last few years. Rodents seem not to be the primary hosts for these organisms, and they are likely to be transmitted by humans. These infections have been named 'post-indigenous diseases' (Weisbroth, 1996).

The presence of infectious agents, even if they are of low pathogenicity, may become a problem if animals from different sources are housed together. This occurs often, as transgenic animals are frequently exchanged between scientists from an almost unlimited number of sources. This is associated with a high risk of introducing different pathogens and thus of causing multiple infections. At present, infections that were common decades ago are re-emerging.

◆◆◆◆◆ IMMUNOCOMPROMISED ANIMALS

Nature has produced quite a variety of mutations affecting the immune system of mice and rats. Some of the deficiencies have been shown to be complex, involving several genes rather than being determined by a single point mutation (e.g. *Prkdc*^{scid}, *Hfh11*^{nu}). Despite phenotypic similarities the genetic basis of various mutations must not be the same. The genetic factors coding for similar phenotypes may act at different developmental stages or differentiation steps (e.g. *Prkdc*^{scid}, *Rag1*/*Rag2*). However, it should be kept in mind that the phenotypic appearance of a mutation might be strikingly altered by the genetic background of the mutant-bearing strain, as has been reported for a large body of examples. While *db*/*db* mice on a C57BL/Ks background (strain of origin) develop an early-onset diabetes that resembles in some respects human non-insulin-dependent diabetes mellitus (type II), the C57BL/6J background has been shown to be diabetes resistant (Coleman, 1978; Leiter *et al.*, 1979). Another example is mice that lack the epidermal growth factor receptor (EGFR). On the genetic background of 129/Sv, mutant fetuses are retarded and die at midgestation; whereas on a mixed background like 129/Sv × C57BL/6 or 129/Sv × C57BL/6 × MF1, fetuses survive until birth and to postnatal day 20, respectively (Sibilia and Wagner, 1995). Mice carrying a null-mutation of the interleukin-2 gene (*IL2*^{tm1Hor}) on the original 129/Ola × C57BL/6 background (129,B6) develop normally during the early postnatal period until about weaning. Thereafter, immunodeficiency becomes evident and the mice die within the next 4 weeks or develop an inflammatory bowel disease (Sadlack *et al.*, 1993). If this knock-out mutation is transferred onto a BALB/c background the lifespan is considerably shortened, with none of the mutants surviving the third week: in C3H/HeJCrl-*IL2*^{tm1Hor} death occurs by 7 weeks and in C57BL/6J-*IL2*^{tm1Hor} by 12–24 weeks of age (Mähler *et al.*, 1996). Such effects of the host genome on the expression pattern of genetically defined single-locus mutations must always be considered, not only when setting up experiments, but also when establishing a new mutation by transferring it onto a given genetic background.

The relative ease of breeding small laboratory rodents also allows for the combination of various mutations and thus of providing experimental animals that are suitable for specific studies. It is not possible to summarize here all the available information and key references on the genetics, pathophysiology, husbandry and reproduction of the abundant natural and induced hereditary immunodeficiencies in rodents – the latter

are growing exponentially in number due to the establishment of new molecular biology techniques. Thus only a rough outline is provided.

Investigators must also be aware that certain environmental factors, both infectious and non-infectious, can lead to transient or persistent suppression or stimulation of the immune system. Such factors (e.g. chlorinated drinking water, tetracycline, infections with MHV or lactate dehydrogenase virus) may complicate research results, regardless of whether the animals are immunodeficient or of wild type (+/+), and should be avoided.

Variants Produced by Nature

Naturally occurring immunodeficient mouse strains express a variety of genetic defects in myeloid and/or lymphoid cell development. These strains have served as, and still are valuable models for, studying immune cell differentiation, mechanisms of transplant rejection, etc. Some of the most commonly used mutants are nude (*Hfh11*^{nu}), severe combined immunodeficiency (*Prkdc*^{scid}), beige (*Lyst*^{bg}), and X-linked immunodeficiency (*Btk*^{xid}). Information about the different variants produced by nature can be found in an ILAR guide (ILAR Committee on Immunologically Compromised Rodents, 1989), Lyon *et al.* (1996) and Hedrich (1990), or by searching for defined mutations in databases such as Mouse Genome Database (http://www.informatics.jax.org) and RATMAP (http://ratmap.gen.gu.se). Tables 4 and 5 give a selection of immunodeficient mutants in laboratory rodents that are often used. Apart from their immunodeficient status (i.e. their inability to eliminate or neutralize foreign substances), some mutants also inherit a failure to discriminate between self and non-self.

In addition to the action of defined genes on the immune function there are several inbred strains or F1 hybrids harbouring genes that confer susceptibility or resistance to infectious or other immune-system-related diseases. As an example, C57BL/6 and related strains succumb to infection with *Streptobacillus moniliformis*, AKR, BALB/c, DBA/2 and other mice survive, while BALB/c mice never show any sign of disturbance, nor even produce antibodies against this organism (Wullenweber *et al.*, 1990).

Variants Produced by Genetic Manipulation

The advent of transgenic rodent technology by transferring and overexpressing foreign genes under the control of specific vectors as well as directed mutagenesis by silencing specific genes has opened up new avenues to study innumerable factors that affect the immune system.

One may search for these either by consulting literature databases, the Mouse Genome Database (MGD; http://www.jax.org/resources; check 'Induced Mutant Resources'), or the Transgenic Animal Database (TBASE; http://www.gdb.org/dan/tbase/tbase.html). Again, as indicated above, identical phenotypes do not necessarily indicate identity of

Table 4. Hereditary immunodeficiencies in mice*

Mutation			Dysfunctions other than immunodeficiency		Care	
Locus	Name	Chromosome	Autoimmunity	None – immune	Special breeding	Special husbandry
bg (*Lyst*^{bg})	Beige	13	–	–	–	–
Dh	Dominant hemimelia	1	–	+	+	–
gl	Grey–lethal	10	–	+	+	–
gld (*FasL*^{gld})	Generalized lymphoproliferative disease	1	+	–	–	–
Hc	C'5 deficiency	2	–	–	–	–
hr	Hairless	14	–	+	+	–
lpr (*Fas*^{lpr})	Lymphoproliferation	19	+	–	+	–
lps	Defective lipopolysaccharide response	4	–	–	–	–
me (*Heph*^{me})	Motheaten	6	+	+	+	–
nu (*Hfh11*^{nu})	Nude	11	+	+	+	+
oc	Osteosclerosis	19	–	+	+	+
op (*Csfm*^{op})	Osteopetrosis	3	–	+	+	+
scid (*Prkdc*^{scid})	Severe combined immunodeficiency	16	–	–	–	+
W (*Kit*^{w})	Dominant spotting	5	–	+	+	–
xid (*Btk*^{xid})	X-linked immunodeficiency	X	–	–	–	–
Yaa	Y-linked immunodeficiency	Y	+	+	–	–

* For references, see Lyon *et al.* (1996) and http:www.informatics.jax.org./mgd.html.

If special breeding regimes are advisable this is indicated by + and indicates, for example, mating of (tested) heterozygotes, continued back-crossing, or even ovarian transplants to histocompatible hosts. Special husbandry (+) means either the need for an SPF environment, for special diets or other environmental conditions; (–) does not imply that, for example, SPF conditions are not required.

Table 5. Hereditary immunodeficiencies in rats, guinea-pigs and Syrian hamsters*

Mutation			Dysfunctions other than immunodeficiency		Care		Ref.
Locus	Name	Chromosome	Autoimmunity	None – immune	Special breeding	Special husbandry	
Rat							
an-2 (b)	Anaemia 1	5	–	+	+	–	Hedrich (1990)
C4	C4 deficiency	20	–	–	–	–	Hedrich (1990)
C6	C6 deficiency	?	–	–	–	–	Hedrich (1990)
ia	Incisor absent	5	–	+	+	+	Hedrich (1990)
l (Lyp)	Lymphopenia	4	–	(+)	–	(+)	Hedrich (1990)
mk	Masked	?	–	+	(+)	+	Hedrich (1990)
op	Osteopetrosis	?	–	+	+	+	Hedrich (1990)
rnu ($Hfh11^{rnu}$; whn^{rnu})	Nude	10	–	+	+	+	Hedrich (1990)
tl	Toothless	?	–	+	+	+	Hedrich (1990)
Guinea–pig							
C2	C2 deficiency	Mhc-linked	(+)	–	–	–	Bitter-Suermann *et al.* (1981) Böttger *et al.* (1986a)
C3	C3 deficiency	?	–	–	–	+	Böttger *et al.* (1985) Böttger *et al.* (1986b)
C4	C4 deficiency	?	(+)	–	–	–	Böttger *et al.* (1986a) Peltier (1982)
Syrian hamster							
C6	C6 deficiency	?	–	+	–	–	Yang *et al.* (1974)
nu	Nude	?	–	–	+	+	Loridon-Rosa *et al.* (1988)

* See footnote to Table 4.

the genes. *Prkdc*^{scid}, *Rag1*$^{-/-}$ and *Rag2*$^{-/-}$ deficient mice, which in many respects are phenotypically alike, have been shown to be different in terms of the genetic factors that control the expression of these immunodeficiencies. On the other hand, silencing of exon 3 of the *whn* gene has produced exactly the same phenotype as in *Hfh11*^{nu} mice, providing evidence that the fork-head transcription factor is responsible for both the nude and the athymic phenotype (Nehls *et al.*, 1996). Table 6 lists a few of the innumerable immunocompromised mutants that have been created in the recent years. PCR protocols that can be used to distinguish between mice carrying an induced mutation (maintained at the Jackson Laboratory) and normal wild-type mice are available on the World Wide Web (http://www.jax.org/resources/documents/imr/protocols/index.html) or through an e-mail inquiry to micetech@aretha.jax.org.

It should be noted that transgenic animals can only be maintained at or be supplied to premises that comply with the national requirements of the respective host country for the use of genetically modified animals.

◆◆◆◆◆ MANAGEMENT OF COLONIES

Housing Systems

The original descriptions of housing systems for small rodents have not lost their principal validity (see e.g. Spiegel, 1976; Otis and Foster, 1983; ILAR Committee on Immunologically Compromised Rodents, 1989), although many refinements have been introduced. In principle, the following different hygienic levels are distinguished: conventional, with no or low precautions; specified pathogen-free (SPF); gnotobiotic and germ-free; presumed infected 'quarantine', and infected. The different hygiene levels require different levels of precaution and presume adequate housing systems, which are used in the opposite safety version for quarantine and infected animals. The housing systems described below have different prerequisites in terms of the construction of the building and equipment. Their running is more or less labour and cost-intensive. The decision about the scientific requirements to be met must be made with respect to international standards.

Conventional

According to 'Good laboratory animal practice' (National Research Council, 1996), climatization of rooms, light cycle, standardized food, special bedding, adequate equipment, prevention of wild mice from entering the animal rooms, food and bedding stores, acceptable animal density and careful handling of the animals are inevitable prerequisites for running a conventional colony. The conventional system should be improved by basic hygienic precautions (e.g. overshoes, overalls, and hand washing). The colony should be monitored regularly in order to detect infections, which may influence the experimental results or the

Table 6. Some transgenic and gene targeted mice*

Mutation		Dysfunctions other than immunodeficiency		Care		
Locus	Chromosome	Autoimmunity	None – immune	Special breeding	Special husbandry	Ref.
β2m	2	–	–	–	–	Koller *et al.* (1990)
Btk		–	–	–	–	Khan *et al.* (1995)
Cd4	6	–	–	–	–	Locksley *et al.* (1993)
Hfh11^{nu} (*whn*)	11	+	+	+	+	Nehls *et al.* (1996)
Tcrα	14	–	+	–	+	Mombaerts *et al.* (1992a)
Tcrβ	6	–	+	–	+	Mombaerts *et al.* (1992a)
Icam1	9	–	–	–	(+)	King *et al.* (1995); Sligh *et al.* (1993) Xu *et al.* (1994)
IL2	3	–	+	–	(+)	Mähler *et al.* (1996); Sadlack *et al.* (1993)
IL4	11	–	+	–	(+)	Kühn *et al.* (1991); Metwali *et al.* (1996) Noben-Trauth *et al.* (1996)
IL6	5	–	–	–	(+)	Kopf *et al.* (1995b)
IL10	1	–	+	–	+	Gazzinelli *et al.* (1996); Kühn *et al.* (1993)
IL12	6	–	–	–	(+)	Mattner *et al.* (1996)
Rag1/Rag2	2	–	–	–	+	Mombaerts *et al.* (1992)
Selp	1	–	–	–	(+)	Bullard *et al.* (1996); Mayadas *et al.* (1993)
Tap1	17	–	–	–	(+)	van Kaer *et al.* (1992)
TgN(Bcl2)22Wehi	?	+	+	–	–	Strasser *et al.* (1991)
TgN(Lck Il4)1315Dbl	?	–	+	–	+	Lewis *et al.* (1993)

*See footnote to Table 4.

embryo-producing capacity. In addition, monitoring means that the risk to other colonies within the animal facility can be better calculated and precautions initiated.

Specified pathogen-free barrier units

By definition according to the international conventions (see pages 117–121, Box 1 and Table 3), specified pathogens cannot be found in a SPF barrier unit. However, no statement on the residual microbiological status is given, implying the possibility of extensive differences from one SPF colony to another (Heine, 1980; O'Rourke *et al.*, 1988; Boot *et al.*, 1996; Rodrigue and Lavoie, 1996). When transferring animals from one SPF to another SPF unit, it should be taken into account that by this action other 'non-pathogenic' microbes or variants may be introduced into the colony which can disturb the microbiological equilibrium, especially in immunodeficient animals (Oshugi *et al.*, 1996).

The SPF level can be established in units of very different size – individually ventilated cages, isolators, or a larger room unit within an animal facility – and it can be run within a certain scale of restriction. In the following we describe the highest standard of SPF, as required for an SPF-breeding unit. A closed area with a strict hygiene barrier system with respect to air supply, materials, food, bedding and personnel (Otis and Foster, 1983; ILAR Committee on Immunologically Compromised Rodents, 1989) is required. After disinfection of the SPF area, high-standard animals can be introduced either directly from a germ-free or gnotobiotic isolator or, when coming from an extramural source, via a mini-isolator (e.g. HAN-Gnotocage) where the filters have been sealed by a foil for the duration of the transfer through a peracitic acid, hydrogen peroxide or otherwise disinfected lock.

A *standardized diet* can be sterilized by X-ray irradiation or by autoclaving. In the case of X-ray irradiation the outside of the package has to be disinfected. If food is sterilized by autoclaving, it has to be 'fortified', i.e. heat labile vitamins have to be added in such an excess that sufficient amounts remain intact after heat treatment. When changing to a new batch, the hardness and the acceptance of the food after autoclaving should be regularly controlled. It should be mentioned that deviations from batch to batch cannot be avoided because of the naturally varying origin of the food ingredients.

Drinking water should be sterilized by heat, filtration ultraviolet (UV) light treatment, but without further precautions bacterial growth is still very rapid in the bottles and also in automatic drinking systems. Therefore, acid (e.g. hydrochloric or acetic acid) should be added to a pH of 3.0–2.5, which will inhibit the growth of microbes, including that of *Pseudomonas* spp. (ILAR Committee on Immunologically Compromised Rodents, 1989). One should note that acidified water may raise problems when vitamins or drugs are to be added. While acidification may change immune functions only marginally, extensive chlorination has been reported to alter the immune response (Fidler, 1997; Herman *et al.*, 1982).

Bedding should be dust-free (<1% dust) and autoclaved after one or two cycles of vacuum/steam exchange. Pregnant females, especially of poorly breeding strains, should be provided with additional nesting material such as autoclaved cellulose towels or nestlets (Sherwin, 1997; Van de Weerd *et al.*, 1997). The recommendations regarding the population density (Weihe, 1978) and the maintenance of biological rhythm (Wollnick, 1989) should be followed.

The microbiological status is to be regularly monitored, sick animals should be removed from the unit and submitted to necropsy/microbiological examination, and sentinels should be regularly checked (see pages 115–117). Single rooms should be emptied, sealed from the remaining unit, cleaned and then disinfected (e.g. with formalin, hydrogen peroxide or commercially available disinfectants), once or twice a year.

The highest risk for the system is, however, the *personnel* entering the barrier unit. They should be well trained (FELASA, 1995) and aware of hygiene risks. The members of the SPF-area staff should be as constant as possible. If staff members have come into contact with rodents outside the SPF area they should not be allowed to enter it until a certain period of time (4–7 days) has elapsed. Members of the staff may be checked regularly, especially after having had an infection (throat and stool specimen). Persons entering the SPF area should shower and wear sterilized clothing inside the barrier system. It must be stressed that within the SPF area strict

Box 2 Double-lock room

For SPF-containment:

1. Stationary position: room and lock doors are closed.
2. Place provisions from the clean floor in the lock; the animal caretaker enters the lock and closes the door.
3. Flush the lock.
4. Opening to the animal room: after closing the inner lock door, flush or disinfect the lock.
5. Working in the room.
6. Opening the inner lock door to the dirty corridor: waste is placed in the lock; the animal caretaker enters the lock and closes the door.
7. Flush the lock.
8. The door to the dirty corridor can now be opened.
9. After closing the door, flush or disinfect the lock.
10. Stationary position.

For experimental use:

Especially for experiments with infectious agents in immunocompromised animals.

hygiene rules must be followed. At least once a week the floor and all equipment should be washed down with a non-volatile formulation of a disinfectant.

If properly managed, such systems may stay 'clean' for many years, although the permanent risk from personnel and technical accidents should not be neglected. In addition, it should be realized that an outbreak of an infection is unlikely to be restricted to a single room (Boot *et al.*, 1996). Therefore, one-way direction animal rooms have been proposed, equipped with a lock to the clean corridor and a lock to the dirty one (see Box 2).

Laminar air flow cabinets

In this design a constant flow of HEPA-filtered air of at least 1.2 f s^{-1} (0.4 m s^{-1}) has to be achieved by mass air displacement within the space to be used for setting up the animal cages (ILAR Committee on Immunologically Compromised Rodents, 1989). Each hindrance within the space, e.g. the cages themselves, may induce whirls and counterflow, which increase the risk of contamination (Thigpen and Ross, 1983). For this reason, exclusively filter covered cages should be used, which protect the animals from cross-contamination within the flow and, in addition, allow protected transfer to a working bench for changing of cages and for experimental manipulation. For low-risk infection experiments, the suckling version may be used. Altogether this housing system is not economical and should only be used in exceptional cases.

Ventilated cabinets

These filter-equipped units, optionally equipped also with a climatization facility, are used to protect small rodents from contamination in the room, and personnel from exposure to dust, allergens, microbes and emissions from the animals (the latter if linked to the exhaust from the room). The uncontrolled status that occurs when doors are opened can be avoided by using filter-covered cages, which will, in addition, protect the animals against cross-contamination within the cabinet and during transmission to the working bench. As in the mass air displacement system, the high airflow over the filter top will prevent bad climatic conditions within the cage. The same equipment switched to negative pressure offers considerable protection in animal experiments involving infectious agents.

Individually ventilated cages

Principal considerations

Microisolator cages (Kraft, 1958; Serrano, 1971) combine the advantages, especially when working with transgenic and immunodeficient strains, of accessibility and isolation at the cage level, and are discussed in more detail below. The original problem encountered with isolator cages was to

combine sufficient ventilation with effective filtration (Lipman, 1992). In fact, it was found that in static (i.e. not forced) ventilation conditions the exchange of air between the interior of the cage and the room was fairly low, causing the humidity and the carbon dioxide and ammonia concentrations to increase to intolerable levels within the cage (Schoeb *et al.*, 1982; Lipman *et al.*, 1992; Choi *et al.*, 1994; Huercamp and Lehner, 1994). In addition, residual air exchange was found to occur mostly via the space between the filter top cover and the cage (Keller *et al.*, 1989), implying a break in the petri-dish barrier. Therefore, animals should not be housed in filter top cages in static conditions other than for short-term transport within a facility. A considerable improvement was achieved by the use of individually ventilated cages (IVC) system. Various systems of this type are now commercially available as a complete rack unit with HEPA-filtered ventilation and exhaust (for a comparison of different systems see Corning and Lipman, 1991; Hasenau *et al.*, 1993; Perkins and Lipman, 1996; Tu *et al.*, 1997). In the IVC system, the environmental conditions within the cages are less variable and less stressful for the animals. Temperature, humidity and ammonia levels comply with or are better than those required by the *Code of Practice for Housing and Care of Animals used in Scientific Procedures* (Home Office, 1989). Although the noise levels are higher than the room background level, they are found to be tolerable (Perkins and Lipman, 1996).

The major advantage of the IVC system with regard to immunodeficient animals is the protection of the animals from airborne contamination at the cage level. We and others have found that mice can be protected against MHV infection by positive-pressure IVCs within a room or rack that is also occupied by infected mice in unprotected cages (Dillehay *et al.*, 1990; Lipman *et al.*, 1993). In addition to protecting animals, the IVC system also reduces the levels of aeroallergens, which may cause health problems for personnel (Hunskaar and Fosse, 1993; Clough *et al.*, 1995) and eliminates pollutants if connected to the exhaust. However, it should be mentioned that, if not properly constructed, the exhaust of the cages can soil channels and obstruct prefilters.

Commercially available IVC-racks are equipped with a ventilation unit that is mounted on the top, on the bottom or separately in the animal room. A further development could be the integration of the IVC system into the room ventilation, allowing ease of accessibility to the machinery. The air supply to the residual room does not need to be ultrafiltered and the air exchange rate can be reduced, saving costs by up to 50% and thus compensating, at least partially, for the high cost of the IVC system (Lipman, 1993; Clough *et al.*, 1995). In the latter context it should be remembered that the animals are exposed directly to fluctuations, especially in the temperature of the climatization machinery, without any compensation from the air of the room.

Running the IVC system at negative pressure helps to protect the environment from contamination by quarantined or infected animals. However, special requirements need to be considered when working with immunocompromised animals (see pages 137–138).

Handling

This is the most critical and most underestimated part of running an IVC system. Principally, three different hygiene levels have to be distinguished: (1) the high sterility level of autoclaved material, diet and water; (2) the room and the outside of the cage; and (3) the inside of the cage, i.e. the animals and their immediate environment. The latter may be different from cage to cage. With regard to manipulation, the protocol in Box 3 is recommended (Homberger, personal communication).

Box 3 Handling of IVCs

1. The laminar flow bench, class II (Biohazard), is switched on.
2. Dilute the sterilization compound (disinfectant)* for gloves. Gloves must be kept moist during the whole procedure.
3. Place an autoclaved filter top cage in the flow bench.
4. The cage to be changed is placed in the bench.
5. The filter tops of both cages are removed and set aside.
6. Sterile diet and water bottle are placed in the clean cage.
7. The animals are transferred to the new cage by using a sterilized forceps.
8. The filter tops are replaced and the cages are removed from the bench.
9. The bench is disinfected occasionally.
10. In the case of infectious animals, the used cage is autoclaved.

* *Recommendation:* Use a very fast-acting sterilization compound, e.g. one based on glutardialdehyde (Chlidox) or chlordioxide (e.g. Chlidox or Alcide). Alcohol and commercially available hand disinfectants are not sufficient.

The procedure is very labour intensive, but this can be compensated for in part by extending the cage changing interval (due to the high ventilation rate, bedding is kept dry and the ammonia level low). Increasing the change interval reduces the stress on the animals. An automatic watering system saves time, but carries a higher risk of contamination due to the interconnection of individual cages and bacterial growth in the pipes.

The IVC system, although expensive to establish and time consuming to run can be used to breed and maintain animals in SPF conditions, and is particularly useful when the structural prerequisites for an SPF unit are lacking and easy access is indispensible for experimental reasons. The negative-pressure version of the IVC system is ideal as a quarantine unit for animals received from different sources and for experiments involving low pathogenicity micro-organisms.

Isolators

Isolators in the positive-pressure version are indispensible for germ-free or gnotobiotic stocks, and in the negative-pressure version as a quarantine station or for high-risk infection experiments. Isolators are made of

flexible polyvinyl film, polycarbonate or stainless steel, the latter two being physically more robust than the first.

In the positive-pressure version, the air supply is equipped with an autoclavable HEPA-filter unit and an exhaust with a valve to prevent contamination due to backflow, or a further HEPA-filter unit. A chemically sterilizable lock is used to connect the interior of the isolator to a supply chamber (Trexler, 1983). Depending on the construction, chemical sterilization of the interconnecting space is required, and this has to be flushed with air from the isolator directly to the exhaust by a connecting tube. Materials are autoclaved in loose packaging within the supply chamber, and the water bottles (screw top, semi-stopped) are sterilized and cooled in the autoclave below the seeding point. Each autoclaving process should be controlled in the supply chamber by means of temperature indicators (paper and/or maxima-thermometer) for immediate validation, and by using a bio-indicator (*Bacillus stearothermophilus*) for the retrograde validation. It should be mentioned that there is some retardation of the heating within the supply chamber with respect to the autoclave chamber, which has to be compensated for by the sterilization process.

Dietary problems may result from the considerable reduction of the nutritional value after thermal sterilization – the latter should be sufficient to kill bacterial spores. Alternatively, an X-ray irradiated diet or, for special investigations (e.g. endotoxin effects in germ-free animals), an X-ray irradiated semi-synthetic diet (Enss *et al.*, 1997) can be used after chemical sterilization of the outside of the package.

In the negative-pressure version, the exhaust air is passed through a HEPA-filter and the lock is used together with the autoclaving chamber for the removal and treatment of waste. This system is used for experiments involving high-risk pathogens.

A combination of both versions, where the inlet and exhaust air are HEPA-filtered, offers the protection of the environment and of the animals. If the protection of the environment is of primary importance, the isolator should be run with an overall negative pressure. The lock and the autoclaving chamber are used for both the sterile supply and the disinfection of waste. The most serious disadvantages of this system are that it is extremely labour-intensive and the difficulty of manipulating the animals within the isolator.

Special Considerations on Immunocompromised Animals

Propagation

The consequences of gene manipulation on susceptibility to disease cannot be predicted fully (e.g. Fernandez-Salguero *et al.*, 1995). Therefore, the aim when creating a gene-manipulated animal should be to maintain the highest possible standards of hygiene, especially during the second part of the procedure, when the foster mothers and the embryos to be reimplanted are being handled, and during the further management of the colonies. Of course this is of special importance when raising immunocompromised animals. In practice, special staff should be available for these tasks. In

addition, different people should be responsible for the manipulation and experimentation on the low restricted side and on the clean side, respectively. The risk of contaminating the clean side via the embryos is low if proper washing is carried out (see page 163).

Adherence to a strict regimen offers the possibility of raising transgenic animals at a level of hygiene that is adequate for immunocompromised strains, thus avoiding time-consuming rederivation. In this context, it should be mentioned that a clear-cut designation of the donor strain, the construct and the ES cell line (if used) should be given according to international rules (Davisson, 1996).

Housing

The microbiological standards outlined above are of special importance for immunodeficient animals because of their high susceptibility to common as well as opportunistic infections (Mossmann, 1992). Immunocompetent animals are able to overcome most infections owing to their immune system; many immunocompromised lines, however, are unable to eliminate the pathogens (Rozengurt and Sanchez, 1993). In addition, when working with immunodeficient animals attention should be directed to the translocation of bacteria from the gastrointestinal tract through the epithelial mucosa and into the organism (Oshugi *et al.*, 1996). The question arises as to whether the SPF standard is adequate for severely immunodeficient animals, or if a more stringent contaminant standard (gnotobiotic, germ free) is necessary. However, it should be taken into account that the immune response may depend on the general prestimulation of the immune system, which is lower in gnotobiotic or germ-free animals. Therefore, caution should be exercised when comparing experimental results obtained in animals maintained at different hygiene levels.

Experiments with infectious agents

Immunocompromised animals in particular should be protected from the environment and, at the same time, the environment should be protected from the infectious agent used in the experiment. As already mentioned, ventilated cabinets equipped with filter hood cages offer a far-reaching solution, but cannot satisfy both these functions unless they are used in combination with a barrier system. The development of sealed IVCs, i.e. biocontainers at the cage level, offers a further improvement in this direction. The highest standard solution available is the use of isolators in the combined version (see page 137).

Mating Systems

As mentioned earlier, the phenotype of a gene governing a state of immunodeficiency – either 'natural', induced or transgenic – may be seriously altered by its genetic background. While most of the established natural and induced mutants have been established in or transferred to an inbred background, many of the most recently developed transgenic and

targeted mutants have a segregated mixed background, which should be back-crossed to more than one defined inbred strain in order to be able to make comparisons with the transgenic or targeted mutant and the modulating effects of different genetic backgrounds. There are many mating systems for breeding rodents (Green, 1981). Unfortunately not all of them can be used either to propagate or to maintain a mutation resulting in immunodeficiency in the bearer. Nevertheless, the major mating systems are: (1) inbreeding by brother × sister matings, thus transferring the mutant/mutated allele to a standard inbred strain background; and (2) propagating the mutation without inbreeding. The latter is used only in those cases where inbreeding is not successful. Assisted reproduction such as *in vitro* fertilization (IVF; Box 4) with embryo transfer (Box 5) might be required. If viability and fertility are reduced, specific measures might be necessary. If the mutant females are unable to mate, or, although being fertile in the sense that they are able to produce functional oocytes are infertile in the sense that they are physically unable to bring offspring to term (as is the case in mice carrying the obese mutation or muscular dystrophy), transplantation of ovaries to unaffected syngeneic or, for example, C.B. 17-*scid* females (Stevens, 1957).

Box 4 In vitro *fertilization of mouse ova*

The protocol described below is a modification of the one reported by Hogan *et al.* (1994).

- *Animals:* 6- to 8-week-old females (superovulated); fertile males.
- *Media:* PB1 (Whittingham, 1971) for embryo collection; Whitten's medium (Whitten, 1971) for culture.

1. Cover Whitten's medium with paraffin oil, preincubate overnight (37°C, 55% CO_2, 95% humidity).
2. 12.0 h after injection of the females with human chorionic gonadotrophin (hCG), kill the males and collect sperm from the epididymis. Suspend sperm in Whitten's medium and incubate for 1.5 h.
3. 12.5 h after injection of the females with hCG, kill the females and collect oocytes in PB1 without removing the cumulus cell mass. Incubate oocytes in Whitten's medium for 1 h.
4. 13.5 h after injection of the females with hCG, add the oocytes to the sperm suspension.
5. 4 h after adding the oocytes, change the medium.

Box 5 Embryo transfer to the oviducts of pseudopregnant surrogate dams

Pseudopregnancy is induced by mating females with vasectomized males of proven sterility, or genetically sterile males, heterozygous for the T(X;16) 16H reciprocal translocation indicated by the Tabby (*Ta*) marker (Lyon *et al.*, 1964), or heterozygous in two pseudo-allelic

(*cont.*)

Box 5 (*cont.*)

variants of the mouse *t* complex (T/tw^2) (Silver, 1985). Females selected to be in oestrus will increase the yield of recipients. Note that, in contrast to mice, it is difficult to produce timed pseudopregnant surrogate rats – neither cervical stimulation with a vibrator nor the application of a vaginal tampon has been very effective. However, vasectomized male rats have provided reasonable results. Copulatory plugs are easy to determine (in rats by means of an otoscope). Induction of pseudopregnancy is timed such that a synchronous (i.e. same chronological stage) or asynchronous (i.e. recipient stage minus 1 day of embryo development) transfer can be performed.

Although embryo transfer may be performed by one operator, two are recommended, one to anaesthetize the recipient, exteriorize the oviduct and the uterine horn, and close the abdominal wound (recipient operator), and the other to load the transfer pipette with the embryos and perform the actual transfer (donor operator). This type of procedure will cause only minimal distress to the recipient female and, therefore, attain much better results.

All cleavage stages from zygotes to morulae can be transferred to the oviduct of pseudopregnant recipients to complete their development with a high rate of success. The timing of pseudopregnancy and developmental stage of the embryo is less critical than in the case of uterine transfers. Best results are achieved if 2- to 8-cell embryos are transferred. With this technique the embryos must have an intact zona pellucida. Normally only unilateral transfers are performed with 5–8 embryos per recipient. If more than 8 embryos are to be transferred to one recipient, they are partitioned and transferred to both oviducts.

The surrogate dam is anaesthetized. After hair clipping and wiping the recipient's lower back with 70% ethanol, the animal is placed under a stereomicroscope with strong incident illumination. An incision about 1 cm in length is made at the level of the ovaries. The skin is slid to either side toward the location of the ovary. The body wall is severed with fine scissors. The ovary, oviduct and proximal end of the uterine horn are exteriorized and fixed with a microwire clamp. The ovarian capsule is then disrupted by means of two No. 5 micro-forceps. To avoid excessive haemorrhage from ruptured vessels (a regular occurrence in rats) one drop of epinephrine solution is applied topically to the bursa ovarica. The infundibulum (always pointing caudally) is located, the transfer capillary inserted and fixed in place with No. 5 micro-forceps, and the embryos deposited by carefully blowing into the mouthpiece. The 2–3 air bubbles drawn up into the pipette to disrupt capillary suction before loading with embryos now serve as an indicator of the amount of medium containing embryos that has been ejected. Withdrawing the capillary the infundibulum should be compressed with the micro-forceps to prevent any reflux and loss of embryos. All exteriorized organs are replaced and the skin incision is closed with wound clips. No suturing of the muscle incision is necessary, providing it has been kept small.

Inbreeding

A unique advantage in working with mice and rats is the availability of standard inbred strains. By using this type of strain, including an F1 hybrid, rather than an outbred stock or a stock with a mixed genetic background, it is possible to eliminate genetic variability as a source of variation. This homogeneity within strains is obtained by continuous brother × sister (B × S), or younger parent × offspring matings for a minimum of 20 generations. After this period, 98.02% of all loci within the genome of either animal of the particular strain should be homozygous. After F12, the remaining heterozygosity within the (incipient) inbred strain will decrease by 19.1% per generation. The increase in homozygosity deviates from the expected value if there is any selective force (inadvertent or intended) towards a certain phenotype or mutation.

Congenic strains

To identify the effects of a particular locus, the use of congenic strains is obligatory. Congenic animals represent attempts of genetic identity with the inbred partner strain, except for the alleles at a single locus. Congenic strains can be produced by certain mating systems, depending on the nature of the differentiating locus, i.e. whether the phenotype can be determined in the heterozygous state and whether the locus affects viability or fertility. Furthermore, as the phenotype of a gene may be altered by other genes of the genetic background, it might be advisable to transfer the variant or mutated allele onto a further standard inbred strain. A detailed description and analysis of the various systems of transferring a mutation/mutated gene onto an inbred background has been presented by Green (1981).

Most of the targeted mutations are induced in embryonic stem (ES) cells, derived from a 129 strain, which are then injected into C57BL/6 blastocysts with a subsequent mating to C57BL/6 followed by B × S or parent × offspring matings. This will result in strains that might be considered as recombinant congenic with an unknown admixture of finally fixed alleles (≥ F20) of the two progenitor strains. It is therefore advisable to propagate the targeted mutation further by back-crossing to C57BL/6 or 129, or another common inbred strain.

If ES cells derived from C57BL/6 or BALB/c are used and targeted ES cells are injected into the blastocysts of the corresponding progenitor strain, at least two cycles of back-crossing should be performed to compensate for possible chromosomal defects acquired by the ES cell line during *in vitro* culture.

The simplest approach for generating a congenic strain is to produce an F1 hybrid from a cross between an animal carrying the allele of interest with the selected inbred partner. The resulting progeny are back-crossed to the inbred partner. This is repeated at least for a further nine back-cross generations. With this scheme, one-half of the unwanted donor genome not linked to the differentiating locus is lost at every generation. Since the advent of PCR-typed DNA-markers which enable identification of the

locus of interest or one closely linked to it, other mating systems are no longer required. If the recessive allele in the homozygous state is lethal or induces sterility, a known heterozygote (as defined by genotyping) is back-crossed to the selected inbred strain. If genotyping is not possible, cross-intercross matings are performed, whereby carriers are identified by the production of mutant offspring. Once identified, the homozygote is crossed with the inbred strain and the resultant progeny are again intercrossed.

There is a statistical probability that mice from an incipient congenic strain developed by using the back-cross system continue to segregate in loci derived from the (induced) mutant donor origin (Fig. 1). The amount of residual genome retained (differential segment) depends on crossing-over events near the locus of interest and thus on the number of back-crosses performed. The probability (P_n) of a contaminant gene of donor origin other than the differentiating gene can be calculated as $P_n = (1-c)^{N-1}$, where N is the number of back-cross generations ($N = 1$ = F1) and c is the probability of crossing over between the differential and residual passenger genes. When $c = 0.5$, i.e. when the passenger gene is not linked to the differential locus, the probability of retaining such an allele is 0.002 after 10 generations of back-crossing. However, if linked genes are considered, one has to expect that after 10 generations the probability of retaining an allele of the donor strain within the 20 cM range is about 13%, within the 10 cM range about 39%. For 'speed congenic' production see

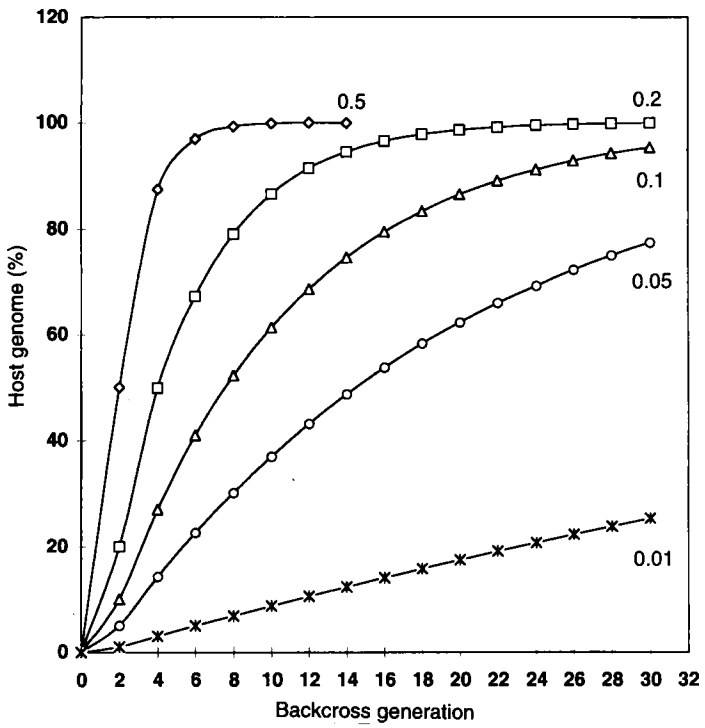


Figure 1. Probability that a certain percentage of host genome has become fixed at various back-cross generations for genes that are unlinked to the differentiating locus ($c = 0.5$), are moderately linked ($c = 0.2$ to 0.005) or tightly linked ($c = 0.01$).

Wakeland *et al.* (in press), who have used computer simulation to model various strategies.

By applying marker-assisted selection protocols, i.e. a genome-wide scan of genetic polymorphisms distinguishing donor and background strain, the production of genetically defined congenic strains is possible within a period of 1.5–2 years. Apparently, with low density marker spacing (25 cM apart) and screening of four litters (only males) at every generation a sufficient back-crossing is achieved after only five generations of back-crossing. In addition, the genome scan allows to identify the chromosomal location of a transgene in *N2* and does provide information on (unwanted) donor-derived regions.

Propagation without inbreeding

Certain mutants cannot successfully be inbred or transferred to a specific inbred background. In these cases the mutation has to be maintained on a hybrid background such as an outbred stock, or descendants of an F1 hybrid. It is thought that these animals with a heterogeneous background are hardier, more productive, faster growing and have a longer life-expectancy. For example, it is extremely difficult to maintain the athymic nude mutation of the rat (*Hfh11*^{rnu}, *Hfh11*$^{rnu\text{-}N}$) on DA and LEW backgrounds.

Many of the targeted mutants are maintained on the variable, mixed background of the ES-cell donor and recipient strain and sometimes another 'prolific' strain or stock. If an immunological mutant cannot successfully be inbred due to effects on viability and fertility, there is no other option but to maintain it on a segregating background or to back-cross the mutant permanently to two different standard inbred strains and to produce homozygous mutant offspring on an F1 background by mating mutant-bearing animals of either strain.

In all instances where research is to be carried out using animals from partially inbred or back-crossed strains or from non-inbred stocks, one should be aware of the genetic variability of these experimental animals and therefore use as controls unaffected (heterozygous and +/+) littermates. If these littermates are not available, F2 offspring derived from the two progenitor genomes provide the closest approximation to the background genotype; F1 hybrids, being genotypically identical, will be the least suitable match.

Genetic Monitoring

Mutations and differential fixation of alleles at early generations of inbreeding may alter the genetic constitution and thus the phenotype of an inbred strain. Many of the phenotypic differences encountered between substrains are due to these factors. Inadvertent outcrossing (genetic contamination) will alter a strain seriously, making its further use for research questionable, since the results are no longer comparable and

repeatable. It is therefore of utmost importance to separate strains that are not immediately to be distinguished by their phenotypic appearance. If, however, due to shortage of shelf space and separate animal rooms one has to co-maintain several strains in one room, regular screenings for the mutant as well as strain-discriminating markers are indispensable.

Proper colony management is the first step towards the provision of authentic laboratory animals (Box 6).

Box 6 Principles of proper colony management

1. During regular handling only one cage at a time should be managed. This will prevent accidental exchange of animals from different cages.
2. Animals that have escaped or dropped to the floor must never be returned to the suspected cage unless the animal can be identified by a non-interchangeable sign such as an ear tag, a tattoo or a transponder signal specifying the animal by strain name (code) and animal number. Traps should be set in larger animal rooms to catch stray animals. Animals caught outside the cage should be killed or isolated.
3. Cages and hoods should be in sufficient condition that no animal can escape or enter another cage, a problem more often encountered in mouse than in rat breeding units.
4. For ease of identification and in order to prevent an inadvertent mix-up, cage tags should have a strain-specific colour code and a strain-specific number (code).
5. Cage tags should always be filled out properly, including the strain name, strain number, parentage, date of birth and generation.
6. If a cage tag is lost, one should not redefine the cage except in the case of definite proof of identity through marked animals within the cage.
7. If at weaning the number of animals is larger than that recorded at birth the whole litter should be discarded or submitted to the genetic monitoring laboratory.
8. If it is inevitable that several strains are housed together, care should be taken to select strains that are easy to distinguish by their coat colour and that will give rise to hybrid offspring different in pigmentation to either parental strain. Strains or substrains that are difficult to differentiate not only by phenotypic appearance but also by laboratory tests must not be kept in the same quarters.
9. Any change in phenotype and/or increase in productivity should immediately be reported to the colony supervisor. The latter change should always be considered suspect for a possible genetic contamination.
10. Regular training programmes on basic Mendelian genetics, systems of mating and the reproductive physiology of the animals maintained should make animal technicians and caretakers conscious of the consequences any mistake will impose on the colonies. Further training should stress the importance of a search for deviants as potentially new models for biomedical research.

As repeated handling of animals during regular caretaking cannot be avoided, there is always the risk of errors. An animal might inadvertently be placed in the wrong cage, or an incorrect entry put on the label. Assigning this type of work to well-trained and highly motivated animal technicians should be a matter of course. The colony set-up and the structuring of nucleus colonies in a single (Festing, 1979) or parallel-modified line system (Hedrich, 1990), pedigreed expansion colonies and multiplication colonies should be self-evident, and strictly monitored. There are several publications dealing with the set-up of colonies for maintenance and large-scale production (Green, 1966; Lane-Petter and Pearson, 1971; Hansen *et al.*, 1973; Festing, 1979). In general, permanent monogamous mating is to be given preference, as this provides a constant colony output with minimal disturbance of the litters during the early postnatal period and by utilizing the chance that females are inseminated at the post-partum oestrus.

The measures required for genotyping a strain have to be adjusted to the specific needs and may depend on the scientific purpose, and on the physical maintenance conditions and laboratory equipment used. Nevertheless, there are specific demands (although unfortunately not stringent rules) on how to authenticate a strain or to verify its integrity.

For any authentication it is necessary to determine a genetic profile that is to be compared with published data (if available), and which allows one to distinguish between (all) strains/stocks maintained in one unit. In general, this profile is composed of monogenetic polymorphic markers, which may be further differentiated by the method of detection into immunological, biochemical, cytogenetical, morphological and DNA markers. Due to the recent rapid development of microsatellite markers (simple tandem repeats (STRs)), these have almost fully replaced the classical genetic markers in routine applications. A large number of primer pairs for mice and rats is available (e.g. through Research Genetics Inc., Huntsville, AL, USA; http://www.resgen.com). Other sources of primers are also available through the World Wide Web (e.g. markers developed by the Wellcome Trust Centre for Human Genetics, Oxford, UK; ftp://ftp.well.ox.ac.uk/pub/genetics/ratmap). However, as with the classical markers it is indispensable to set up a genetic profile representing a random sample of the genome, which should be evenly spaced on the chromosomes, and which will allow one to discriminate between all strains maintained per separate housing unit. Unfortunately this information is only partially available and not yet compiled in an accessible database. Conditions for PCR amplification and electrophoretic separation of the amplicons are described in Box 7. These conditions deviate from those provided primarily with respect to electrophoretic separation. As there is only one amplification protocol it could be necessary to adjust the temperature conditions and Mg^{2+} concentration for each microsatellite marker. For routine screening, separation on agarose gel and visualization by ethidium bromide will suffice. If separation of the amplicons is insufficient in agarose, one should run a polyacrylamide gel electrophoresis (PAGE; see Box 7). As radioactive labelling is with ^{32}P using a kinase reaction, and since the isotope half-life is relatively short, a silver staining procedure is recommended (Box 7).

Box 7 Protocol for characterizing and typing STRs with one primer set per PCR reaction

The amounts needed are based on the quantities calculated for a single reaction well plus a small excess. To work out the amount of the various reaction mixtures, simply multiply by the number of DNA samples to be tested:

1. Add mineral oil (30 µl) to each well of a 96-well plate (not necessary when using hot bonnet).
2. Pipette 5 µl template DNA (20 ng μl^{-1}) into each well.
3. Centrifuge the plate briefly to collect the template in the bottom of the wells; apply the plate at 96°C for 3 min, and then at 4°C until adding the mastermix.

PCR:

1. Preparation of mastermix:

Forward primer (6.7 µM)	0.25 µl
Reverse primer (6.7 µM)	0.25 µl
dNTP (1.25 mM/dNTP)	2.5 µl
10× PCR buffer	1.5 µl
H_2O	4.75 µl
Taq-polymerase (5 U μl^{-1})	0.15 µl
Total	10.00 µl.

2. Transfer 10 µl mastermix to each well.
3. Centrifuge and run the PCR reaction.
4. Add 3 µl BFB to each well.
5. Load on a 3% Nu sieve or a 1.5% Sigma Type II Agarose gel in 1 × TBE; alternatively, use polyacrylamide gel (PAGE) (see below).
6. Run the electrophoresis for 3–4 h at 70 V.
7. Stain with ethidium bromide (in case of PAGE, use silver staining; see below)

PCR programme

1. 3 min at 94°C 1×
2. 15 s at 94°C
3. 1 min at 55°C } 30×
4. 2 min at 72°C
5. 7 min at 72°C 1×

PAGE:

- 6–7.5% polyacrylamide/bis(acrylamide) in 1× TBE (13 × 16 cm) (Sambrook *et al.*, 1989).
- Spacer 0.4 mm.
- Probe volume 2–6 µl (gel loading buffer type 2 (Sambrook *et al.*, 1989).

(*cont.*)

Box 7 (*cont.*)

Silver staining:

Note: Use only twice-distilled water for all solutions and washings and prepare all solutions directly before use.

- Fixing: 30 min, 10% acetic acid (v/v); 3 × 2-min wash in H_2O bidest.
- Staining: 20–30 min, 0.1% $AgNO_3$ (w/v), 0.037% formaldehyde. (The time of staining depends on the gel concentration and gel thickness.) Rinse for 5–10 s with H_2O bidest.
- Developing: 2–15 min in 2.5% Na_2CO_3; add 0.037% formaldehyde; add 0.002% sodium thiosulphate (solution). (These solutions must have a temperature of 4–5°C, maximum 10°C, and pH 12.0. *Note:* Do not use any plastic container for developing procedure.)
- Desilvering: in 10% acetic acid.

Nevertheless, the classical markers are still relevant and may need to be verified. They may even allow for a faster and less expensive phenotyping.

Immunological markers

Immunological markers comprise cell surface markers, such as: major histocompatibility antigens (*H2* in the mouse and *RT1* in the rat); lymphocyte differentiation antigens; red blood cell antigens; minor histocompatibility antigens; allotypes (immunoglobulin heavy-chain variants), which can be determined by Trypan blue dye exclusion test (see pages 189–204); flow cytometry (see pages 23–57); immunodiffusion, ELISA (see pages 621–650); and immunohistochemistry ELISA (see pages 257–286), using specific antibodies. The availability of antibodies depends on the specific marker and the species, with a broader spectrum available for mice. These markers may also be demonstrated by applying molecular biology techniques, such as oligotyping of major histocompatibility complex (MHC) class I and class II genes by reverse transcriptase PCR (RT-PCR) and dot blot and reverse dot blot hybridization, respectively (see pages 148–149; for other specific markers check also: http://www.informatics.jax.org/mgd.html).

Biochemical markers

These are the classical electrophoretic markers, which almost have been replaced by STR typing unless a specific allelic expression is to be verified in an experiment. Apart from qualitative enzyme and protein polymorphisms, enzyme differences, such as Car2 in mice, may have to be quantified to differentiate between homozygous *Car2°/Car2°*, heterozygous *Car2°/+* and wild-type (+/+) mice.

Box 8 Identification of MHC class I and II alleles by oligotyping

***H 2D* alleles of laboratory mice**

The homologies of the MHC class I genes in the murine species do not allow identification of each *H2* class I allele using one specific oligonucleotide. Three allele-specific oligonucleotides are required to identify one of the *H2D* alleles. Two of these oligonucleotides are used as a specific pair of primers to predifferentiate the alleles, independently of the gene loci by enzymatic amplification of a relevant RNA fragment. It is advisable to use a DNA polymerase that shows reverse transcriptase activities, as this will reduce the time and cost of typing. In the case of the *H2D* gene, the complementary sequences from the specific forward and backward primers are located in the highly polymorphic exons 2 and 3 of the class I mRNA, so that the resulting amplicons include further polymorphic areas (Fig. 2). The final identification is performed by hybridization of an amplified fragment with the third oligonucleotide that corresponds to an allele-specific sequence within the amplicon.

1. Isolate total RNA from tissue.
2. Predifferentiate *H2D* alleles by RT-PCR using allele-specific primer pairs. The optimal annealing temperature ($T_{A_{opt}}$) of the primer pairs should be calculated by the nearest neighbour method, which takes into consideration the enthalpy and entropy from each base pair (Rychlik and Roads, 1989; Rychlik *et al.*, 1990). It is recommended that 'touch down PCR' be run over all cycles in order to increase the stringency of the PCR conditions.
3. The amplified fragments are visualized by agarose gel electrophoresis and ethidium bromide staining.
4. Identify *H2D* alleles by dot blot hybridization using allele-specific oligonucleotides (Fig. 3). The dot blots are prepared by spotting an aliquot of the denaturated PCR products onto a nylon membrane. The DNA is immobilized by UV cross-linking or by baking the membrane.
5. The allele-specific oligonucleotides are labelled with radioactive or non-radioactive markers.
6. The hybridization procedure is carried out under absolute stringent conditions. The dissociation temperature (T_d) of oligonucleotides should be calculated by the nearest neighbour method, while the optimal hybridization temperature ($T_{hyb_{opt}}$) must be determined empirically. DNA/DNA hybrid detection depends on the type of oligo-labelling.

MHC class II RT1.B$_\alpha$ and RT1.B$_\beta$ alleles of inbred rat strains by oligotyping

The differentiation of the MHC class II alleles of laboratory rats is based on the same principles as the *H2D* typing. Prior to hybridization using specific oligonucleotides, a RT-PCR is needed to amplify the

relevant allele fragments. In contrast to the MHC class I genes, the non-orthologous MHC class II genes diverge clearly in their nucleotide sequences within a species (Wakeland *et al.*, 1990). For this reason the two *RT1.B* genes can be differentiated by RT-PCR using gene-specific primer pairs flanking the highly polymorphic exon 2. The final identification is followed by reverse dot blot hybridization of the amplicons. In this case the hybridization partners change their function. Compared to *H2D* typing the advantage of this procedure is that there is only a limited number of protocols because the hybridization temperature for each $RT1.B_{\alpha}$ or $RT1B_{\beta}$ allele is identical.

1. Isolate total RNA from tissue.
2. Differentiate the $RT1.B_{\alpha}$ and $RT1.B_{\beta}$ genes by RT-PCR using gene-specific primer pairs.
3. The optimal annealing temperature ($T_{A_{opt}}$) of the primer pairs should be calculated by the nearest neighbour method as for the predifferentiation of *H2D* alleles (Rychlik and Roads, 1989; Rychlik *et al.*, 1990). It is recommended that a 'touch down PCR' be run over all cycles to increase the stringency of the PCR conditions.
4. The amplicons should be labelled with Dig UTP during the PCR, to reduce the number of protocols.
5. The amplified fragments are visualized by agarose gel electrophoresis and ethidium bromide staining.
6. Identify the $RT1.B_{\alpha}$ and $RT1.B_{\beta}$ alleles by reverse dot blot hybridization using two sets of specific oligonucleotides for the $RT1.B_{\alpha}$ and $RT1.B_{\beta}$ alleles (Fig. 4).
7. The oligonucleotides should correspond to the hypervariable areas of exon 2 of the $RT1.B_{\alpha}$ and $RT1.B_{\beta}$ genes. It is important that all oligonucleotides of one set hybridize at about the same temperature.
8. The reverse dot blots are prepared by spotting the tailed oligo dT oligonucleotides onto a nylon membrane with a dot blot apparatus. The oligonucleotides are immobilized by UV cross-linking with the membrane.
9. The $RT1.B_{\alpha}$ or $RT1.B_{\beta}$ allele can be identified by its hybridization pattern (Fig. 4).

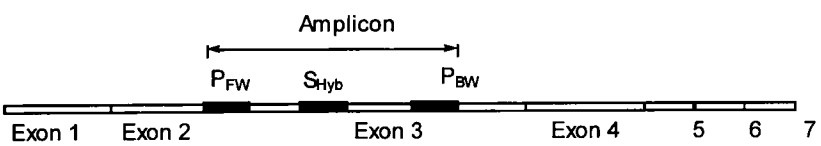


Figure 2. MHC class I mRNA. P_{FW}, forward primer; P_{BW}, backward primer; S_{hyb}, oligonucleotide.

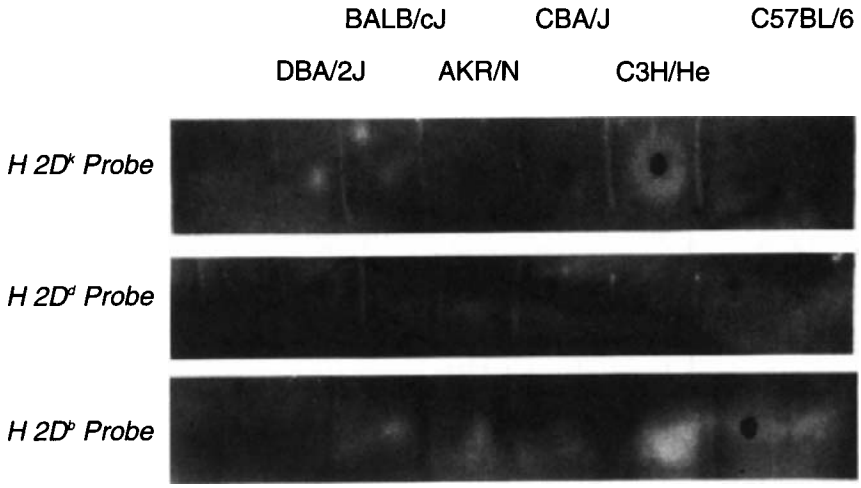
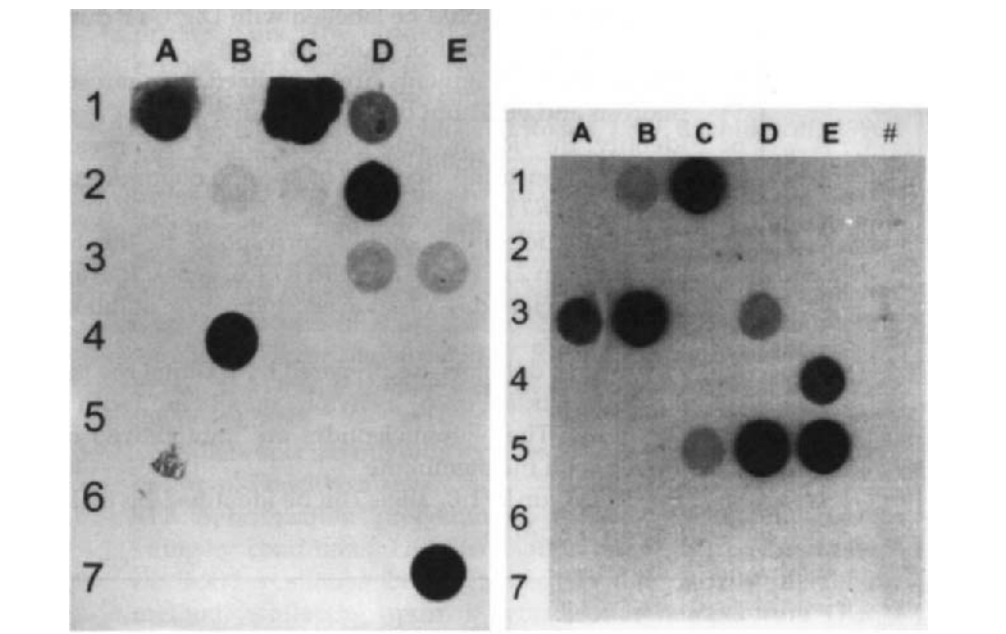


Figure 3. Identification of *H2D* alleles by RT-PCR and hybridization using allele-specific oligonucleotides.



(left) (right)

Figure 4. Identification of the $RT1.B_{\beta}^{u}$ (left) and $RT1.B_{\alpha}^{u}$ allele (right) by reverse dot blot hybridization. Columns A to E mark the variable areas in the exon 2 of the *RT1.B* gene. The possible oligonucleotides for each variable area are fixed on lines 1–7 of the membranes. (#) A column that contains only one oligonucleotide specific for a 6 bp insertion in $Rt1.B_{\beta}$ alleles.

Cytogenetic markers

These play a role if mice carrying numerical variants and structural aberrations of chromosomes are being maintained (e.g. the T(X;16)16H translocation; see page 139).

Morphological markers

These include coat colour and pelage variants, but also markers controlling skeletal abnormalities and metabolic and neurological deviants. A reasonable amount of coat colour genes show pleiotropic effects on the immune system. For example, beige, which is an allele of the lysosomal trafficking regulator (*Lyst*), is demonstrated by its pigment-reducing effect (if not hidden due to an epistatic effect of albino, *c/c*), by a prolonged bleeding time because of a platelet storage pool defect (20 min in homozygous $Lyst^{bg}$ vs 6 min in unaffected wild-type or heterozygous controls), and by abnormal giant lysosomal granules detectable in all tissues with granule-containing cells (histological sections, cytocentrifuge preparations of PBL).

As the determination of a genetic profile is time-consuming and expensive it is only feasible as an initial check. In the case of a variable segregating background, genetic profiling is pointless; the typing results may only assist in determining the degree of heterogeneity, but may provide hints about modifying genes if the stock is being inbred and almost homozygous.

PCR protocols used to demonstrate specific mutant genes are provided for the respective marker in MGD (http://informatics.jax.org; check: Genes, markers and phenotypes, see RFLP/PCR polymorphism) and for induced mutations (maintained at the Jackson Laboratory) that can be distinguished from normal wild-type mice on the World Wide Web (http://www.jax.org/resources/documents/imr/protocols/index.html) or can be obtained through an e-mail inquiry to micetech@aretha.jax.org. Moreover, information on RFLP polymorphisms as determined by a Southern blot (Sambrook *et al.*, 1989) using a specific probe is also provided in MGD, if applicable and available.

Simple measures are needed to distinguish between those strains that are co-maintained and those that clearly identify an outcrossing event. A critical subset of the markers (i.e. the least amount of differentiating marker for a given strain panel) used to authenticate the strains maintained will provide reasonable information about the genetic quality of a strain. Unfortunately, with each strain added to a unit the number of markers in the critical subset increases. Critical subsets need to be verified at regular intervals (every 3–6 months). The intervals and the number of animals to be tested depends on the number of strains co-maintained and the size of each colony.

One of the most powerful pieces of information about an inbred strain is the demonstration of isohistogeneity. This is best demonstrated through skin grafting, which is simple to perform, although time-consuming because of an observation period of about 100 days (Box 9). In specific immunodeficient mutants (e.g. $Hfh11^{nu}$, $Prkdc^{scid}$, $Rag1^{tm1}$, $Rag2^{tm1}$) a direct demonstration of isohistogeneity is impossible as these animals are

incapable of mounting an allorecognition response. This can be circumvented by transferring grafts from these immunodeficient animals to their syngeneic background strains.

Box 9 Orthotopic tail skin grafting

Animals aged 6–9 weeks serve best as recipients for orthotopic tail skin grafting. The animals to be grafted are anaesthetized with a volatile or injectable anaesthetic (ether inhalation or ketamine hydrochloride supplemented with xylazine). The tail is scrubbed with antiseptic solution. The animals are placed in ventral recumbency with the tail pointing towards the operator. Thin sheets of skin are sliced with a scalpel (blade No. 11 or 20) towards the tail base. The cut should be as deep as possible but should not sever the dorsal tail artery or vein. Occasional bleeding may be stopped by one or two drops of epinephrine solution (1 : 1000). For a regular reciprocal circle two (several) grafts are taken per animal at the same time, providing the graft bed to receive the grafts from two (several) other animals. Therefore, the method requires meticulous sample preparation. All grafts have to be of the same size (approximately 6×2 mm in mice and 8×3 mm in rats). The grafts are placed in Petri dishes on saline-drenched filter paper, and the excised graft beds are covered with gauze sponges moistened in saline until grafts are transferred. Then the appropriate grafts are positioned on the prepared beds such that the direction of hair growth is reversed. Excessive fluid and blood is removed by pressing with dental rolls. The grafts are then fixed with liquid surgical dressing and secured by glass tube slipped onto the tail and fixed with a tape. The tube should not exert any pressure on the tail base, as this could lead to a severe oedema due to blocking of the venous drainage.

After recovery from anaesthesia the animals are housed separately on large wooden shavings or on cellulose sheets for the first 2–3 days until the tube is removed. Regular bedding may be scooped into the tube to absorb moisture and scrape off the grafts (at least the proximal ones) when the glass tube is removed.

Grafts are inspected daily for 2 weeks from day 8–10 onwards. Thereafter a graft appraisal once weekly until day 100 post-grafting will suffice. Genetic outcrosses cause the graft to be rejected in an acute fashion and are thus immediately detected.

Technical failures become evident at the first appraisal. Either the grafts are recorded to be dislodged or to be ripped off with the tube. Orthotopic tail skin grafts are sometimes removed by the animal itself during the first week by grooming. This technical failure (of the first set grafts) is evidenced by the presence of an eschar at or before day 7. Technical failures are in the range of 5–10%.

Scoring may vary from laboratory to laboratory, either by fully describing the graft appearance or by defining scores. Skin grafts should be recorded as rejected if and when they are reduced to a scar.

(cont.)

Box 9 (*cont.*)
Other signs, such as a pasty appearance of the graft epidermis or less than 50% of its original surface being intact, may also be taken as an indication of graft rejection. With the latter two types of assessment the median survival times of allogeneic grafts will be shorter by a few days. Specific scores separately defining the graft by size and outward appearance will ease the appraisal of skin grafts in a large number of animals (Hedrich, 1990).

If there is any doubt about the success of graft acceptance, regrafting is essential (usually within 2–3 weeks). In the case of true incompatibility (not technical failure), the second set graft will be rejected in a hastened and more pronounced manner. Grafts are considered to have been accepted when the entire graft has healed completely with no clear indication of contraction (< 25% shrinkage). The final assessment after 100 days must take account of previous ratings.

In the case of immunodeficient animals incapable of rejecting an allograft, skin is grafted to the immunocompetent background strain. If the mutation itself is not acting antigenically and no graft is rejected this is proof of isohistogenicity within the strain and with the genetic background.

In order to reduce and simplify routine monitoring procedures, techniques that are fast, reliable and cost-effective are preferred. Random amplification of polymorphic DNA by PCR (RAPD) meets these demands. With this method (Williams *et al.*, 1990) anonymous stretches of genomic DNA are amplified using arbitrarily designed single short primer sequences of about 10 nucleotides. Recombinant inbred strains of mice have been distinguished by using this method (Scott *et al.*, 1992), as have various inbred strains of rats including MHC congenics (D. Wedekind and H.J. Hedrich, unpublished).

Box 10 Differentiation of inbred rat strains by PCR using a random primer (RAPD)

1. Genomic DNA is prepared according to the method described by Miller *et al.* (1989) from ear or from blood, using a DNA extraction kit (Quiagen, Hilden, FRG).
2. RAPD primers with arbitrary nucleotide sequences can be purchased from, e.g., Roth, Karlsruhe, FRG. The 10-mer primers are characterized by their GC content (60%, 70%, 80%).
3. The PCR is carried out in a 25-µl reaction volume containing reaction buffer, dNTPs, one random primer, genomic DNA and DNA polymerase. Amplification is performed on a thermal cycler. The conditions for the PCR must be strictly standardized.
4. The amplified fragments are separated by horizontal gel electrophoreses on a 1–1.4% agarose gel in 1 × TBE (0.1 M Tris/borate, 2 mM EDTA), at constant current (100/80 V, 70 mA) for 5–6 h. The PCR products are visualized with ethidium bromide (Fig. 5).

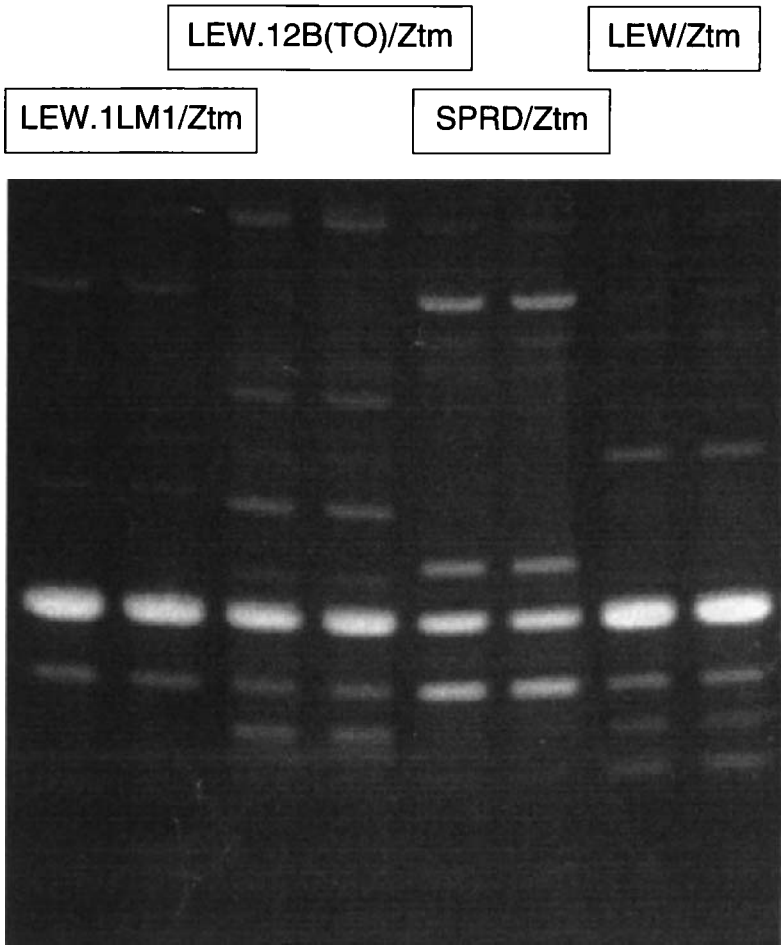


Figure 5. The figure shows the differentiation by RAPD of three congenic inbred rat strains (LEW.1LM1/Ztm, LEW.12B(TO)/Ztm and LEW/Ztm) and one inbred rat strain (SPRD/Ztm).

Another recently developed technique is the demonstration of amplified fragment length polymorphisms (AFLP™, Keygene n.v., Wageningen, Netherlands) (Zabeau and Vos, 1992). The technique is based on the combined use of restriction enzymes and selective PCR primers. Multiple polymorphisms are simultaneously visualized without the need of prior information on genomic sequences. DNA is cleaved into fragments using a set of two restriction enzymes: a rare cutter and a frequent cutter. Adapters are ligated to the ends of the restriction fragments. Adapters that stick to the site of the rare cutter carry a biotin label. Biotin-carrying fragments are isolated by binding to streptavidin beads, resulting in an enormous reduction in the number of fragments. Only fragments containing a rare-cutter end on one side and a frequent-cutter end on the other side, or rare-cutter ends on both sides, will remain in the fragment pool. Subsequently, a further selection will be performed by

PCR using selective primers. The PCR primers overlap the adapters and the restriction sites and are provided with a specific extension at their 3'-ends causing the further selection. The resulting amplicons are separated by PAGE. The AFLP pattern reflects multiple polymorphic markers of presence/absence type, i.e. dominant/recessive markers (Otsen, 1995).

◆◆◆◆◆◆ CRYOPRESERVATION AND REVITALIZATION OF LINES

The high costs of animal care and maintenance often makes it difficult (for a researcher) to maintain strains that are no longer actively used. Furthermore, many individual research colonies are microbiologically contaminated, so that virus-free facilities are reluctant to import mice from unknown sources. Therefore, the freezing of preimplantation embryos is considered to be the proper means to cope with the multiplicity of strains of mice and rats presently available, to serve as a safeguard against loss, to allow for eradication of infections if the embryo transfer is performed under aseptic conditions onto barrier maintained surrogate dams, and to reduce the costs for valuable strains presently not used. Despite certain improvements, the freezing of murine embryos is a time-consuming and cost-effective task. While outbred stocks and hybrids in general respond to superovulation by gonadotrophins (see protocols in Box 11) with a high ovulation rate, inbred strains show a rather variable response. In addition revitalization results also vary substantially on a strain by strain basis, and strongly depend on the skill of the personnel. Therefore, it has not been possible to preserve as many strains recently developed by molecular genetic methods as necessary. Freezing of sperm, if sufficiently efficient, could assist in this task. As with sperm freezing the protocols for *in vitro* fertilization (IVF) (see Box 4) also need to be improved.

The original technique of embryo freezing as described by Whittingham *et al.* (1972) and Wilmut (1972) requires a controlled slow freezing and slow thawing procedure with dimethylsulphoxide (DMSO) or glycerol as the cryoprotectant. Since this first description of successful freezing of eight-cell mouse embryos, various modifications in the use of cryoprotectants, freezing methods and freezing of other developmental stages have been reported (for an overview see Hedrich and Reetz (1990)).

Freezing of embryos at a slow speed (0.3–0.8°C min^{-1}) to –80°C permits the embryos to undergo progressive dehydration, thus preventing intracellular ice-crystal formation. Thawing has then to be slow (about 8°C min^{-1}) in order to allow the blastomeres to rehydrate without deleterious side-effects. This is the method established and used at The Jackson Laboratory, Bar Harbor, ME, USA.

The procedure of freezing embryos at a low rate (0.4°C) to a subzero temperature of only –30°C to –40°C with subsequent immersion in liquid nitrogen requires thawing at about 300–500°C min^{-1}. The latter method is

less time-consuming, less expensive, and more practical types of freezer are available. However, embryos frozen by a 'fast' technique are thought to be in a metastable state and very slight alterations during warming and cryoprotectant removal might damage the embryos seriously (Leibo, 1981). The success of revitalization not only depends on strain/species and on the freeze–thaw technique (Rall *et al.*, 1980; Rall, 1981), but also on the embryo transfer and the skill of the practitioner. The two-step freezing and vitrification procedures established in the authors' laboratories, which have been shown to give reasonable results with two-cell embryos from mice and rats, are given in Boxes 12 and 13.

The selection of embryo donors depends on the type of strain to be cryopreserved. In the case of an outbred stock the group of revitalized breeding pairs required to build up a new colony should be genetically equivalent to their colony of origin, i.e. the genotype distribution within both populations should be equivalent. Furthermore, mating of close relatives must be avoided to ensure that the coefficient of inbreeding is kept at a low level. A random sample of breeders collected from the source colony may fulfill this requirement and serve as donor parents. In practice this can be realized if only embryos with different ancestors are frozen in one single cryotube (subsequently termed 'embryo batch') and if a number n of independent batches is used to rederive a new colony. In dealing with inbred strains it is to be differentiated whether a foundation colony is to be restocked or whether the frozen embryos should serve in future as breeders of an expansion colony. The former have to be derived from a single pedigreed breeding pair, preferably originating from the foundation colony. Restocking of an expansion colony also calls for pedigreed embryos, but these can be derived from different donors as strict brother × sister mating is not mandatory. In the case of congenic (CR) and mutant, as well as most transgenic or knock-out lines, it is the primary objective to maintain the differential or mutant/mutated gene. For this purpose a pool of embryos (descending from different donors) may suffice. After rederivation, however, one to four back-cross cycles to the background strain are required.

It has been shown in mice as well as rats that all preimplantational stages can be revitalized successfully upon freeze–thaw procedures. For long-term storage eight-cell stages have been recommended, while two-cell stages were considered to be less suitable. Results obtained in the authors' laboratories (see also Mendes da Cruz, 1991) show that frozen–thawed two-cell embryos can be revitalized at a comparable rate. The two-cell embryos are transferred into the oviducts of day-1 surrogate dams, eight-cell embryos may be transferred into the oviducts of day 1–2 surrogate dams, or into the uterus after a 24 h culture period.

One embryo batch (inbred strain) derived from a single pedigree donor pair may be regarded as a prospective breeding nucleus, if one fertile breeding pair is obtained upon revitalization. Assuming an average revitalization rate of 20% (fertile breeders), one embryo batch should contain a minimum number of 10 embryos to obtain at least one breeding pair with a 50% chance of revitalization (Table 7). According to these figures, 100 cryopreserved embryos is to be regarded as the lower

Table 7. Minimum number of embryo batches needed to obtain at least one B × S breeding pair as progenitors for a new nucleus*

No. of embryos per batch	Expected no. of weanlings after revitalization	No. of batches needed (probability for ≤1 B × S pair)	
		99.0%	99.9%
10	2	6.7	10.0
15	3	3.5	5.0
20	4	2.2	3.3

* Based on an average revitalization rate (weaned and reproductive for inbred strains of about 20%).

limit to provide a safe backup of a strain. Nevertheless, a safe backup of a strain is affected not only by the size and the number of embryo batches frozen, but also by the revitalization rate significantly differing between strains as well as according to the skill of the staff in embryo transfer techniques. For routine embryo banking, therefore, ≥20 embryo batches per strain, each containing 10–20 embryos, will be sufficient to guarantee a safe backup.

Effective superovulation protocols thus are crucial. The average rate of embryo batches per hormone-treated female (≥10 two-cell embryos per batch per female) amounts to about 30% (range 10–43%). For eight-cell embryos this rate drops to 20–25% (H. J. Hedrich and I. Reetz, unpublished data). If a strain is refractory to superovulation (as it is for certain inbred strains and even non-inbred transgenic stocks), the embryos are obtained from normal mated donor females.

Embryos forming an embryo batch are frozen together in a freezing container. Various types of container are in use, such as 2-ml polycarbonate tubes with screw caps, glass or polypropylene ampoules, and plastic straws. When sterilizing heat-labile embryo containers with ethylene oxide, one has to consider the cytotoxic effect of the absorbed retained gas. Containers sterilized by this method must not be used until a sufficient post-sterilization aeration (approximately 3 weeks) has been completed (Schiewe *et al.*, 1985). There are, however, straws available that withstand heat sterilization. To avoid mixing up embryo batches, each container must be permanently marked with the strain name and strain code. This is facilitated by using a hand-driven printing device. In addition, each freeze run must be monitored by means of a temperature recording, and its reliability should be monitored by using a vitality test of an additional embryo batch of an F1 hybrid or outbred stock highly responsive to superovulation. After the freeze run the cryocontainers should be properly placed at defined locations/compartments within the embryo repository in a properly controlled liquid nitrogen refrigerator. It is important to keep full records, which should include the conditions of the freeze run (type, cryoprotectant used, etc), a strain description, and an identification and storage location. The physical conditions of the freezing procedure, including results of viability tests obtained from the particular control batch, are required as documentation of a correct freezing technique and to provide further information on the thawing procedure to be applied. For each stock

in the repository a description of the strain or mutant, with particular information about phenotype, reproductive performance and strain history, should be kept on a file. Information concerning identification requires complete pedigree information, such as parentage, genotype, generation, the code number of the embryo batch, the number and developmental stage of embryos frozen, and a precise storage location.

The funds needed to run an embryo bank have to cover the personnel expenditure (at least one scientist and one technician), the cost of basic equipment (investment with 10 years amortization) and the running costs. The estimated cost per year is about US$ 90 000–100 000. The cost of maintaining a breeding nucleus of one strain under specified pathogen-free conditions is about US$ 4500–5500. Thus cost equivalence is achieved if approximately 20 strains have been deposited in the repository and are no longer maintained as vital breeding nuclei in the animal quarter.

Sperm freezing, although not well established, could assist in all cases where animal-holding space is limited and strains do not respond well to superovulation. This primarily applies to colonies of mice bearing mutations or transgenes. Recent attempts at sperm freezing (Table 8) associated with IVF are promising.

Box 11

Superovulation protocol for the mouse:

The protocol given below is that described by Whittingham (1971).

- *Animals:* females aged 6 weeks to 4 months.

1. *Day –2, 16.00 h:* inject 5–10 iu pregnant mare's serum gonadotrophin (PMSG).
2. *Day ±0, 16.00 h:* inject 5 iu human chorionic gonadotrophin (hCG), mate to males.
3. *Day +1, morning:* check for the presence of a vaginal plug.
4. *Days +1 to +4:* collect preimplantatory embryos.

Superovulation protocol for the rat:

The protocol given below is a modification of that described by Rouleau *et al.* (1993).

- *Animals:* adult females, at least 59 days old.

1. *Day –4, 08.00 h:* inject 40–60 µg luteinizing hormone releasing hormone (LHRH).
2. *Day –3, 17.00 h:* load the osmotic minipump with follicle stimulating hormone (FSH), so that 6.8 mg FSH is delivered daily. (The pump is kept in sterile saline at room temperature until use in order to reach the nominal steady state value.)
3. *Day –2, 08.00 h:* implant the osmotic pump.
4. *Day ±0, 16.00 h:* inject 30 iu human chorionic gonadotrophin (hCG), mate to males.
5. *Day +1, 08.00 h:* plug control and vaginal cytology.
6. *Days +1 to +5:* collect preimplantatatory embryos.

Table 8. Cryopreservation of mouse spermatozoa

	Songsasen *et al.* (1997)	Sztein *et al.* (1997)	Dorsch and Hedrich (unpublished)
Animals	F1 hybrid	F1 hybrid	Inbred strains
Freezing medium	0.3 M raffinose, 0.2 M glycerol, egg yolk	18% raffinose, 3% skim milk (Nakagata and Takeshima, 1992)	18% raffinose, 3% skim milk (Nakagata and Takeshima, 1992)
Freezing procedure	At slow speed to –20°C, then transfer to LN_2	Transfer to –120°C, then transfer to LN_2	5 min on dry ice, then transfer to LN_2
Thawing procedure	Fast	Fast	Fast
Dilution of freezing medium	One step (1:10), centrifugation	One step (1:10), centrifugation	One step (1:10)
Sperm motility after thawing	Approx. 30%	Approx. 80–85%	Approx. 50%
In vitro *fertilization:*			
Fertilization ability	Approx. 30%	Approx. 85–90%	Approx. 80–85%
Developmental ability	>30%		25–30%
Implantation/offspring	30–35%	37%	Approx. 10%

Box 12 Two-step freezing of embryos

The procedure for freezing embryos at a low rate to a temperature between –30°C and –40°C following the conventional protocol with subsequent immersion in liquid nitrogen has certain advantages. For instance it is less time consuming, less expensive, and more practical types of apparatus are available or may be designed.

Consistent results have been obtained in the authors' laboratory by applying a two-step freezing technique. For freezing, plastic straws are used instead of other containers because they can be better marked and almost no embryos get lost during manipulation. The straws are loaded, with the embryos being placed in the centre of the medium column. In a programmable automatic ethanol cooling bath the embryos are equilibrated at 0°C in freezing medium with 2.0 M propanediol as the cryoprotectant for 5 min, and then cooled to –6°C at a rate of 1°C min^{-1}, seeded (induction of extracellular ice crystal formation by touching the straw at the air–medium interface with a metal rod precooled in liquid nitrogen) and then slowly cooled to –32°C at a rate of 0.4°C min^{-1}, held for about 5 min at –32°C, and transferred directly to the liquid nitrogen refrigerator. The straws are always handled horizontally to keep the embryos in position, until seeding is finished.

Thawing at a rate of about 300°C min^{-1} is achieved by warming the straws at room temperature for about 40 s (Renard and Babinet, 1984; Mendes da Cruz, 1991).

Box 13 Vitrification of embryos (quick freeze/fast thaw procedure)

There are a number of reports that mouse and rat embryos survive freezing after rapid cooling by directly plunging into liquid nitrogen. This quick freeze/fast thaw procedure requires the use of a highly concentrated aqueous solution of cryoprotectants. At sufficiently low temperatures, these solutions become so viscous that they turn into an amorphous state without any formation of ice. This process has been termed 'vitrification'. Most groups use glycerol (3.0–4.0 M) as a permeable and sucrose (0.25–1.0 M) as a non-permeable cryoprotectant. Before freezing the embryos must be dehydrated. At temperatures below 4°C embryos can tolerate exposure to a concentrated solution of cryoprotectants and the associated osmotic dehydration. The following method, based on the one reported by Rall and Fahy (1985), has been shown to give reasonable results in mice and rats.

The vitrification solution (VS1, pH 8.0) is composed of a mixture of 20.5% DMSO (w/v), 15.5% acetamide (w/v), 10.0% propanediol (w/v), 6.0% polyethylene glycol (w/v) in PB1 (mice), or TCM 199 supplemented with 20% heat-inactivated rat serum (sTCM, rats). Embryos are equilibrated at about 0°C (on ice) in four steps at different

(*cont.*)

Box 13 (*cont.*)
concentrations of VS1. Each equilibration step with 12.5%, 25%, 50% and 100% VS1 is exactly timed for 10 min. Then the embryos are transferred to a straw (e.g. Minitüb) containing VS1. Immediately thereafter the straw is sealed and exposed to –196°C (liquid nitrogen), care being taken to keep the straw in a horizontal position during all manipulations.

Although this method does not require an elaborate biofreezer and appears to be rather simple, it has not yet replaced the more conventional techniques. The conditions for pre-dehydration and cryoprotectant removal require further optimizing. Post-thaw survival is variable for the different developmental stages of the embryos, and may depend on the type, concentration, temperature and equilibration time of the cryoprotective solution used for vitrification. In contrast to these requirements, dehydration and removal of cryoprotectant is less critical for the survival of embryos frozen by the two-step method as used routinely in the authors' laboratory (Box 12).

The requirements for thawing embryos are defined by the freezing procedure and the cryoprotectants used. The manipulation depends on the cryocontainer used. In tubes and ampoules a few embryos regularly get lost because they stick to the wall, whereas with straws all embryos are usually recovered.

The straw containing the embryo batch to be revitalized is removed from the liquid nitrogen container. Both tips holding the sealing bulbs are cut off and the straw is attached to a syringe filled with air. When all ice crystals have disappeared (after about 40–45 s), the cryoprotectant solution containing the embryos is gently flushed into an equal amount of PB1 (mice) or sTCM (rats) to reduce the concentration of the cryoprotectant by 50%. After two further stepwise elutions (25%, 12.5%), always with a 10-min equilibration, the embryos are put through five washes in sterile medium, and held for up to 30 min. This permits the embryos to recover from osmotic distress. Embryos that appear to be morphologically unimpaired by microscopic inspection are selected for immediate transfer to pseudopregnant recipients.

◆◆◆◆◆◆ MANAGEMENT OF INFECTED COLONIES

'Natural Infections'

Quarantine/infections

Animals with an unknown microbiological status have to be kept in isolation until the examination is finished. This mostly concerns animals received from other institutions. The state of isolation should be the same

as for infected animals. Because, in general, a broad range of gene-manipulated stocks, which are potentially infected with different pathogens, has to be accepted from outside further isolation from each other should be accomplished. Although ventilated cabinets, IVCs or isolators may be used, the need for rederivation is obvious. The same is true for animals with an unwanted microbiological status which are a risk to the whole facility.

Rederivation

Hysterectomy

As shown for most infections, the vertical transmission of viral, bacterial and parasitic pathogens can be avoided by this procedure. The protocol originally recommended by Trexler (1983) is depicted in Box 14.

The most difficult part of this procedure is to achieve timed pregnancy, especially in poorly breeding strains. This method is recommended if embryo transfer cannot be performed due to lack of equipment and trained personnel, or eventually in the case of a donor strain that is refractory to superovulation. Hysterectomy has the additional risk of intrauterine vertical transmission of micro-organisms, which may be higher in immunodeficient than in immunocompetent animals.

Box 14 Hysterectomy

1. Mate foster mother (outbred or hybrid strain) in the clean area overnight; check for vaginal plug.
2. 24–48 h later, mate animals of the microbiologically contaminated strain; check for vaginal plug.
3. Install the dip tank filled with low-odour disinfectant before the expected date of birth of the foster mother.
4. Shortly before delivery, kill the pregnant dam of the strain to be rederived by cervical dislocation; carry out hysterectomy under aseptic conditions.
5. Transfer the uterus to the clean side through the disinfectant (38°C).
6. Wash the uterus intensively in physiological saline, and develop the pups.
7. As an extra safety precaution, the pups may be dipped again in disinfectant and washed again in physiological saline.
8. After gentle massage with a swab to induce spontaneous breathing and after warming up, transfer the pups to the nest of the foster mother after disposing of her own offspring.
9. If coat-colour discrimination is possible, one or two of the foster mother's pups may be retained to assist in the induction of lactation.

Embryo transfer

Embryo transfer has been shown to interrupt most vertically transmitted infections of viral, bacterial or parasitic origin, with the exception of germ line transmitted retroviral infections. The integrity of the zona pellucida is of decisive importance, as shown for MHV infection (Reetz *et al.*, 1988). The hygiene status of the foster mother should be of the highest level, especially when a new breeding unit is to be established. For routine procedures, two-cell stage embryos may be the most suitable, because fertilization is no longer in question and a higher number of embryos can be collected than at later stages. The animals are mated overnight without or after previous superovulation (for details see Box 11) (Reetz *et al.*, 1988; Hogan *et al.*, 1994; Schenkel, 1995). The latter method allows synchronization of mating, and generally induces production of higher numbers of embryos than by normal mating. The embryos are flushed from the oviducts of plug-positive mice on day 1.5 and washed at least four times in large volumes of media (approximately 2 ml) at a different location before being transferred to the clean area where implantation into the oviducts of pseudopregnant recipients is performed by other staff. Pseudopregnancy is induced by mating with either surgically sterilized or genetically sterile males (see Box 5).

Embryo transfer offers specific advantages over hysterectomy: it avoids the risk of intrauterine vertical transmission of infections; it allows easier timing, especially in the case of superovulation; and it allows cryopreservation of surplus embryos. For special applications we have developed a method for flushing embryos *in vivo*, allowing the use of embryo donors for successive reflushing or 'normal' breeding. This procedure requires extreme skill and cannot be recommended for routine manipulation.

Furthermore, new strains shipped as cryopreserved embryos can be transferred to surrogate dams with the specific SPF status, avoiding time-consuming quarantine and rederivation procedures. Moreover, accidental infection during shipment can be avoided.

Therapeutic treatment

In general, the administration of drugs influences the outcome of animal experiments and cannot be considered as a substitute for improving hygiene standards. The success of treatment depends on several criteria: a correct diagnosis, and consideration of species-specific toxicity, adverse reactions, optimal dosage and regimen of application, accompanying hygiene procedures, etc. Unfortunately, recommended dosages often refer to man or larger animals. For extrapolation to small rodents allometric parameters should be used, which increase the body weight ratio by a factor of approximately 6 and 12 for rat and mouse, respectively, in comparison to man (for a review see Morris, 1995). In addition, the half-life of drugs is, in general, reduced, thus requiring more frequent administration for an effective level of drug to be maintained.

In general, the treatment of parasitic invasions has to be accompanied by hygiene procedures (e.g. chemical and physical disinfection, change of cage). Some commonly used antiparasitic drugs are listed in Table 9. Chemotherapeutic and antibiotic treatment of infections may induce resistance, overgrowth of other bacterial species (Hansen, 1995), or adverse reactions by altering the gut flora (Morris, 1995). Commonly used antibacterial treatments are listed in Table 10 (see also Hawk and Leary, 1995). It should be stressed, however, that the use of therapeutic drugs will reduce, but only occasionally eliminate, parasites or micro-organisms.

Preventive therapeutic treatment may be of help in providing a better chance of transferring quarantined and accidently infected animals. In immunocompromised animals drugs are used to suppress opportunistic infections, especially those of human origin.

Experimental Infections

General precautions

The safe operation of an animal laboratory is one of the main management responsibilities. Housing infected animals requires precautions to prevent transmission of micro-organisms between animal populations and, in the case of zoonotic agents, to humans. The zoonotic risk arising from naturally infected rodents is low because most rodent pathogens do not infect man. Only a few agents like LCMV, Hantaviruses or *Streptobacillus moniliformis* have the potential to cause severe infections in humans and might be prevalent in colonies of laboratory rodents. Severe disease outbreaks in humans associated with infected colonies of laboratory rodents have been reported (Bowen *et al.*, 1975; Kawamata *et al.*, 1987), and therefore safety programmes are necessary to prevent laboratory-associated infections and infections transmitted by laboratory animals.

Experimental infections are more likely to pose a risk for humans. A broad spectrum of infectious agents can be introduced accidentally with patient specimens, and many laboratory animals are still used for infection experiments. In general, health precautions are very similar for clinical or research laboratories and for animal facilities. In many cases, however, an increased risk may arise from experimentally infected animals due to bite wound infections or when pathogens are transmissible by dust or by aerosols.

A number of recommendations exist from federal authorities for microbiological laboratories, aimed at the prevention of infection of laboratory personnel. Many programmes were developed in response to evaluations of laboratory accidents. Most laboratories have written control plans that have been designed to minimize or eliminate risks for employees.

Reduction of the risk of disease transmission can be achieved by very general procedures which are common practice in most well-run facilities housing animals behind barriers. Only major points can be discussed

Table 9. Treatment of common parasites (in combination with hygienic measures)

Generic name	Trade name	Application	Dose	Reference
Ectoparasites				
See table in Weisbroth (1982)				
Ivermectin*	Ivomec (Merck & Co)	Topical spray	0.2–10 mg ml^{-1}	Hirsjärvi and Phyälä (1995)
Endoparasites†				
Piperazine citrate		Drinking water (for 12 weeks, every 2nd week)	0.2%	Maess and Kunstyr (1981)
Fenbendazole‡	Panacur (Hoechst)	Diet‡ several months	100–150 ppm in diet or 25–50 mg kg^{-1} body weight	Strasser and Tiefenbach (1977)
Ivermectin*	Ivomec	Topical spray 2 ml/cage	1 mg ml^{-1}, 2 ml/cage, once weekly for 3 weeks	Le Blanc *et al.* (1993)
		Drinking water	2.9–4.0 mg kg^{-1} for 4 days, 3-day pause, 5 cycles	Klement *et al.* (1996)
Ivermectin–piperazine (combined)		Drinking water	7000 ppm, 2.1 mg ml^{-1}, alternately every 2 weeks for several months	Lipman *et al.* (1994)

For disinfection especially in the case of parasite eggs and oocysts, a chlorocresol formulation has proven valuable (Neopredisan, Menno-Chemie Vertrieb GmbH, 22850 Norderstedt, Germany). For additional drug dosages, see Hawk and Leary (1995).

* Toxic in young mice (Scopets *et al.*, 1996).

† Especially *Syphacia obvelata* and *Aspicularis tetraptera*.

‡ Diet can be autoclaved without substantial loss of efficacy.

Table 10. Selected antibiotic therapies for small rodents

Disease/species	Anti-infective	Application in drinking water	Dose	Reference
Pasteurellosis/mouse	Enrofloxacin (Baytril)	For at least 30 days	25.5–85 mg kg^{-1}	Goelz *et al.* (1996)
Mycoplasma/rat	Oxytetracycline	For at least 5 days*	3–5 mg ml^{-1}	Harkness and Wagner (1983)
	Tylosin	For 21 days	5 g l^{-1}	Carter *et al.* (1987)
Hepatitis–typhlitis/ mouse (*Helicobacter hepaticus*†)	Amoxicillin‡	For 4 weeks – young mice	50 mg kg^{-1}	Russell *et al.* (1995)
	Amoxicillin‡ Metronidozole Bismuth	For 2 weeks	200 mg l^{-1} 138 mg l^{-1} 37 mg l^{-1}	Foltz *et al.* (1996)
Pneumocystosis/mouse, rat	Sulphamethoxazole–trimethoprim (Borgal; Cotrim K, Ratiopharm)	For 3 weeks	200 mg–1 g l^{-1} 40–200 mg l^{-1} (5 ml l^{-1})	H. J. Hedrich (unpublished) Rülicke (personal communication)
Tyzzer's disease (*Clostridium piliforme*)/ mouse, rat, rabbit	No antibiotic therapy recommended; rederivation Special disinfectant required§			

For additional drug dosages see Hawk and Leary (1995).

* Drinking water should not be acidified; addition of 1.35 g l^{-1} potassium sorbat prevents growth of yeast.

† Especially immunodeficient mice.

‡ Toxic for hamsters and guinea-pigs.

§ Chlorocresol (Neopredisan)

here; more details on general laboratory safety are given in many textbooks on clinical microbiology (Gröschel and Strain, 1991; Burkhart, 1992) and in general recommendations for housing of laboratory animals (CCAC, 1980; Bruhin, 1989; Kunstyr, 1988b; BG Chemie, 1990; National Research Council, 1996).

Education is an important part of an effective safety programme. All safety instructions should be in written form and must be readily available at all times.

The first point must be adherence to safety procedures and proper behaviour, such as use of personal protective clothing. Prohibition of eating, drinking, smoking, handling of contact lenses and the application of cosmetics in the laboratory are other basic rules, as is the separation of food storage refrigerators from laboratory refrigerators. The most likely route of infection is direct contact with contaminated animals or materials. Micro-organisms do not usually penetrate intact skin. The risk of infection can therefore be reduced by repeated hand decontamination and by decontamination of surfaces or contaminated instruments.

Working with infectious agents should not be permitted in cases of burned, scratched or dermatitic skin. Needles and other sharp instruments should be used only when necessary, and handling of infected animals should be allowed only by experienced and skilled personnel, to prevent bite wounds. Working in safety cabinets helps to avoid inhalation of infectious aerosols and airborne particles which are easily generated in cages when animals scratch or play. Other procedures that might bring organisms directly onto mucous membranes are mouth pipetting and hand–mucosa contact. Both must be strictly forbidden.

Most animal facilities are constructed in such a way that proper cleaning and disinfection can be performed easily, which helps to control infectious animal experiments. In contrast to clinical laboratories, there is usually no wooden furniture and sufficient space is provided to allow access for cleaning and disinfecting whole rooms or areas.

Microisolator cages are often used in animal facilities for transportation within the facility in order to avoid exposure of humans to allergens. Such cages, too, help to reduce the risk of spreading micro-organisms during transportation.

In most animal facilities containment equipment (microisolator cages, isolators) is used if immunosuppressed animals have to be protected from the environment or if infected animals might be a hazard to humans or other animals. Experiments with infectious agents will usually be conducted in separate areas that fulfil all safety requirements such as ventilation (negative pressure in laboratories to prevent air flow into non-laboratory areas) or, better, in isolators which represent the most stringent containment system. For safety reasons, containment is generally necessary if animals are artificially infected with pathogenic micro-organisms. Various systems can be used, depending on the properties of the agents (e.g. pathogenicity, environmental stability, spreading characteristics). In the case of low pathogenic organisms, microisolator cages might be sufficient. The risk of infection during handling is reduced if all work with

open cages is conducted in changing cabinets or in laminar flow benches. Individually ventilated cages operating with a negative pressure are more suitable than microisolators to prevent spread of micro-organisms if they are handled properly. The highest level of safety can be achieved by using a negative-pressure isolator. If handling through thick gloves is not possible, handling of animals can be performed in safety cabinets that can be docked directly to the isolator.

An important part of safety programmes in laboratories, and especially in laboratory animal facilities, is waste management. In contrast to radioactive or chemical waste, infectious waste cannot be identified objectively. In many cases judgement of whether waste from animals that are not experimentally infected is infectious or not is dependent on the person in charge. There is, however, no doubt if animals have been infected experimentally. In such cases the presence of a pathogen allows evaluation of the risk, which depends on the virulence and the expected concentration of an agent together with the resistance of a host and the dose that is necessary to cause an infection. The risk of pathogen transmission is increased by injuries with sharp items such as needles, scalpels or broken contaminated glass. Segregation of such sharp items and storage in separate containers is necessary to keep the risk of infection to a minimum.

Infectious waste from animal houses (bedding material, animal carcasses) can be submitted to chemical or thermal disinfection, but incineration and steam sterilization are the most common treatment methods. Incineration has the advantage of greatly reducing the volume of treated materials. The usually low content of plastic material in waste from animal housing and the high percentage of bedding material (e.g. wood shavings) resulting in a high-energy yield make incineration the method of choice.

Safety levels

Classification of micro-organisms

Four different safety levels have been established (Centers for Disease Control/National Institute of Health, 1993), which consist of combinations of laboratory practices and techniques, safety equipment and recommendations for operation of laboratory facilities. The classification of an organism or parts of it (DNA, toxin) is based on various factors such as the host spectrum, virulence for healthy humans and animals, minimal infectious dose, mode of transmission, epidemiological situation (prevalence in a given population), availability of antibiotics, vaccines or other treatments, and tenacity (Table 11). The recommended levels represent those conditions under which the organism can ordinarily be safely handled. Sometimes, more stringent practices may be necessary when specific information is available to suggest that virulence, pathogenicity, vaccine and treatment availability or other factors are altered. For example, hantaviruses are typical BL-3 pathogens. Many researchers

Table 11. Examples of micro-organisms belonging to different biosafety levels

BL1	BL2	BL3	BL4
Viruses			
Phages	Hepatitis B virus	Eastern equine encephalitis virus	Marburg virus
Tobacco mosaic virus	Herpes simplex virus	Herpes B virus	Ebola virus
	SV 40	Human immunodeficiency virus	Smallpox
	Parvovirus B19	Yellow fever virus	Lassa fever virus
	Vacciniavirus		Foot and mouth disease
	Rabies virus		African swine fever
	Mouse adeno virus		
Bacteria			
Arthrobacter sp.	*Borellia burgdorferi*	*Bacillus anthracis*	None
Bacillus subtilis	*Clostridium tetani*	*Brucella melitensis*	
Erwinia sp.	*Escherichia coli*	*Chlamydia psittaci*	
Lactobacillus acidophilus	*Enterococcus faecium*	*Coxiella burneti*	
Micrococcus luteus	*Listeria monocytogenes*	*Mycobacterium tuberculosis*	
Oligella urethralis	*Mycoplasma pneumoniae*	*Mycobacterium leprae*	
Pseudomonas fluorescens	*Pasteurella multocida*	*Pseudomonas mallei*	
Streptomyces sp.	*Salmonella enteritidis*	*Rickettsia prowazekii*	
	Staphylococcus aureus	*Yersinia pestis*	
	Vibrio cholerae		
Fungi			
Aspergillus niger	*Aspergillus fumigatus*	*Blastomyces dermatitidis*	None
Alternaria alternata	*Candida albicans*	*Coccidioides immitis*	
Malassezia furfur	*Cryptococcus neoformans*	*Histoplasma capsulatum*	
Saccharomyces cerevisiae	*Microsporum canis*	*Paracoccidioides brasiliensis*	
Rhizopus oryzae	*Trichophyton mentagrophytes*		
Parasites			
Apathogenic trypanosomas	*Leishmania donovani**	*Leishmania donovani*†	None
	Eimeria sp.		
	Trichinella spiralis		

* Work without vector.

† Work with vector.

consider hantaviruses BL-4 agents when inoculated into laboratory animals, especially into rats, since there is clear evidence of aerosol transmission from infected animals. The safety instructions are applicable for working with organs, tissues or cells that contain or may shed microorganisms. For example, many cell lines have been immortalized by SV40 virus and may shed this pathogen. Such cells, like the virus, should be handled by using biosafety level 2 practices.

The biosafety level assigned to an agent is based on activities typically associated with the manipulation of quantities and concentrations of infectious agents required to accomplish its identification. If activities require larger volumes or higher concentrations or manipulations which are likely to produce aerosols, additional personnel precautions and increased levels of containment are indicated. Details on all aspects of biosafety have been published by the Centers for Disease Control/National Institute of Health (1993) and BG Chemie (1990, 1991a,b,c, 1992).

- *Biological safety level I (BL-I)* applies to the use of characterized microorganisms not known to cause disease in healthy human adults. BL-I organisms are, for example, attenuated viruses that are used for the production of life vaccines (e.g. polio vaccine), or viruses that are apathogenic for man and animals. Life vaccines for animals containing viruses that may be pathogenic for humans may keep their pathogenic properties for humans. Therefore, a Newcastle disease vaccine, although apathogenic for poultry, is classified as level 2. Other level I pathogens are many plant viruses (e.g. tobacco mosaic virus) and viruses of bacteria and fungi (phages). All bacteria that do not multiply in warm blooded organisms, saprophytes and bacteria that have been used for the production of foodstuffs (e.g. lactobacilli) or the preservation of vegetables are also classified as class I. Fungi that do not infect healthy humans (even if they have the potential to infect immunocompromised hosts) are classified as level I (e.g. *Saccharomyces cerevisiae, Malassezia furfur, Aspergillus niger*).
- *Biological safety level 2 (BL-2)* is used for work involving agents that represent a moderate hazard for personnel and the environment, for farm or wild-living animals, or for plants. This level is applicable to clinical, diagnostic, teaching and other facilities in which work is done with a broad spectrum of agents that are present in a population and are associated with human or animal disease of varying severity. Most vertebrate viruses and a broad spectrum of bacteria (e.g. *E. coli, Staph. aureus, Clostridium tetani, Vibrio cholerae*) are classified as BL-2 organisms. Fungi that may infect healthy humans or animals (e.g. *Candida albicans, Trichophyton mentagrophytes, Microsporum canis, Aspergillus fumigatus*) and for which efficient drugs are available are also classified as biosafety level 2. Classification of parasites is in many cases dependent on additional factors that may have impact on the risk. Most pathogenic parasites (protozoans, trematodes, nematodes, mites and insects, including lice and fleas) are classified as BL-2. For some protozoal parasites, the primary laboratory hazard arises from transmission by arthropod vectors. For such parasites (e.g. *Trypanosoma cruzi, Leishmania donovani, Plasmodium falciparum*) working is only classified as BL-2 if vectors necessary for transmission are not used. For other

parasites a different risk may arise from larval stages or from adult parasites. For several trematodes the most serious risk arises from larval stages (e.g. *Fasciola hepatica, Dicrocoelium* sp., *Paragonimus* sp., *Opisthorchis* sp., *Schistosoma* sp.). Working with larvae, therefore, requires BL-2 practices, whereas working with adult worms is classified as BL-1. In contrast, working with *Echinococcus* sp. should be conducted under BL-2 conditions only if it is restricted to hydatid cysts, whereas working with adults requires a higher safety level.

- *Biological safety level 3 (BL-3)* is used when working is necessary with indigenous or exotic agents that may cause serious or potentially lethal disease as a result of autoinoculation or ingestion or with a potential for aerosol transmission. Among viruses, yellow fever virus, human immunodeficiency virus (HIV) or herpes virus B are classified as BL-3 pathogens. Only few bacterial species (*Mycobacterium tuberculosis, Yersinia pestis*, Rickettsiae, *Pseudomonas mallei, Coxiella burneti, Brucella melitensis, Chlamydia psittaci*) or fungi (*Blastomyces dermatitidis, Coccidioides immitis, Histoplasma capsulatum*) pose a serious risk to laboratory workers or animals or a moderate risk to the population, and may therefore be classified as BL-3. Few parasites are classified as BL-3 organisms if working includes use of vectors (e.g. *Trypanosoma cruzi*, several *Leishmania* species like *L. donovani, Plasmodium falciparum*). In the case of *Echinococcus* species (e.g. *E. granulosus, E. multilocularis*), working with adult worms may be classified as BL-3, whereas BL-2 is applicable if working is restricted to hydatid cysts.
- *Biological safety level 4 (BL-4)* practices are applicable for work with highly contagious and pathogenic or exotic organisms that may cause lethal infections, for which there is no available vaccine or therapy and which may be transmitted by the aerosol route. Additional agents with a close relationship should also be manipulated at the BL-4 level. Examples are Lassa fever virus, Marburg virus, Ebola virus, or smallpox. Of the animal pathogens, rinderpest, foot and mouth disease and African swine fever should be considered as class 4 pathogens. At present, no bacterial pathogens, fungi or parasites are classified as BL-4.

Safety precautions

- *Biological safety level 1 (BL-1)* requires basic laboratory facilities and the use of standard laboratory practices. No additional safety precautions are necessary if animals are infected with BL-1 pathogens.
- *Biological safety level 2 (BL-2)* requires level 1 practices plus additional measures such as the wearing of laboratory coats and protective gloves. Access is only allowed for persons having specific training in handling pathogenic agents (and technical staff, if necessary); public traffic is not permitted. No protective clothes should be used outside the BL-2 area and all clothes must be autoclaved before washing. Biohazard warning signs must be posted at the entrance door. Food is not allowed to be stored. All persons working in a BL-2 unit should inform supervisors on specific incidents that might influence their resistance to pathogenic micro-organisms (e.g. pregnancy, immunomodulation) as well as on bite or scratch wounds from infected animals.

Persons who are at increased risk of acquiring infection are not allowed in the animal room. All infectious waste must be decontaminated before leaving a BL-2 unit.

Clinical specimens (blood, body fluids, tissues) that may contain micro-organisms pathogenic for humans should be handled using BL-2 practices. Standard precautions include the use of a biological safety cabinet or a biohazard hood when working with any clinical material. These cabinets are the most commonly used primary containment devices in laboratories working with infectious agents. Biological safety cabinets offer the additional advantage of protecting the clinical specimens from extraneous airborne contamination.

- *Biological safety level 3 (BL-3)* requires level 2 facilities and practices supplemented by controlled access to the laboratory and use of special laboratory clothing and partial containment equipment (e.g. a biological safety cabinet). Work surfaces are decontaminated after any spill of infectious material or at least once a day. Entering a class 3 area is possible only via a lock system (two self-closing doors) that strictly separates the area from adjoining rooms. Windows must be sealed or constructed in such a way that they cannot be opened. Access is allowed only for authorized and trained persons who have been instructed in the specific risk situation and whose presence is required. All persons should be supervised by competent scientists who are experienced in working with the agents handled in the laboratory. Technical staff need to be accompanied by skilled persons. Laboratory personnel should be immunized against the agents handled or potentially present in the laboratory.
- *Biological safety level 4 (BL-4)* requires even more strict safety practices than BL-3. BL-4 units are usually located in a separate building. Exhaust air must be HEPA filtered. Entrance doors are usually supplied with access control systems and a lock system (three rooms) with a ventilation system that guarantees that the air stream is flowing into the BL-4 area. Laboratories must be separated from common areas in such a way that access is restricted to authorized persons and is impossible for non-authorized persons. A logbook must be used, indicating the date and exact time of entry and exit. The laboratories have to be disinfected before access of other persons (e.g. technical staff) is allowed. Working alone is not allowed. The laboratory worker must be completely protected from aerosolized infectious materials, which is accomplished by working in a class III biological safety cabinet or a full-body, air-supplied positive-pressure personnel suit. All persons have to take a decontaminating shower before leaving the laboratory. A double-door autoclave and a pass-through dunk tank must be available for decontaminating materials passing out of the laboratory.

Housing systems and operational practices

Biosafety criteria for housing vertebrates have been defined in the USA by the Centers for Disease Control (1988) for biosafety levels 2 and 3, and later for all 4 biosafety levels (Centers for Disease Control/National Institute of Health, 1993). Specific regulations for housing infected

animals according to different safety levels also exist in other countries (e.g. for Germany see Gentechnik Sicherheitsverordnung Anhang V). Therefore, only general comments are given here.

Laboratory animal facilities may be organized in different ways. Sometimes, animal facilities are extensions of laboratories and are managed under the responsibility of a research director. Large research institutions, companies or universities often have centralized laboratory animal facilities that are managed by laboratory animal specialists. They are usually separated from laboratories or institutes. Such facilities usually easier fulfil the legal requirements (animal welfare, safety) due to a more proficient management and specialized personnel, and their size. Centralized animal facilities are usually multipurpose with a number of animal species or strains that are used for a variety of different experiments (short or long term) for different scientific disciplines (e.g. toxicology, immunology, biochemistry, pharmacology, teratology, surgery). Several housing systems (conventional units, barrier unit, isolators) or microbiological quality standards (infected, pathogen-free, gnotobiotic) can be found in large facilities (for more details, see pages 130–137). Therefore, strict separation of animals used for different experiments (studies of infectious or non-infectious disease) or purposes (production and breeding, quarantine) is usually self-evident, not only for safety reasons, but also in order to avoid research complications or influences between experiments. Traffic flow in centralized animal facilities is usually reduced to a minimum, thus minimizing the risk of cross-contamination. Such facilities are usually constructed in a way that proper cleaning and personal hygiene is facilitated. Bedding material from animal cages is removed in a way that formation of aerosols is avoided, in order to minimize the risk of allergies and to reduce the risk of airborne transmission of pathogens. Use of solid bottom cages helps to reduce dust formation and is absolutely necessary if experimentally infected animals are housed. The whole facility must be constructed in a way that escape or theft of animals is impossible.

In general, biosafety levels recommended for working with infectious materials *in vitro* and *in vivo* are comparable. Some differences exist, because the activity of the animals themselves can introduce new hazards by producing dust or aerosols, or they may traumatize humans by biting and scratching. Therefore, the Centers for Disease Control/National Institute for Health (CDC/NIH) (1993) established standards for activities involving infected animals, designated 'animal biosafety levels' (ABSL) 1–4. These combinations describe animal facilities and practices applicable to work on animals infected with agents assigned to the corresponding BL1 to BL4.

Housing animals of ABSL-1 is usually no problem if an animal facility as well as operational practices and the quality of animal care meet the standard regulations (CCAC, 1980; Bruhin, 1989; ILAR, 1997). In contrast to experiments with non-infectious materials, additional hygiene procedures should be applied, such as decontamination of work surfaces after any spill of infectious material and decontamination of waste before disposal. Persons who may be at increased risk of acquiring infections

should not be allowed to enter rooms in which infected animals are housed.

Additional practices are necessary for ABSL-2. Careful hand disinfection is necessary after handling live micro-organisms. All infectious waste must be properly disinfected (preferably by autoclaving), and infected animal carcasses should be incinerated. Cages and other contaminated equipment are disinfected before they are cleaned and washed. Whenever possible, infected animals will be housed in isolation to avoid the creation of aerosols. Physical containment devices are not explicitly required by the CDC/NIH (1993) for ABSL-2. Microisolator cages are not recommended because they do not reliably prevent aerosol formation and transmission of micro-organisms. They should only exceptionally be used for housing, and must be placed in ventilated enclosures (e.g. laminar flow cabinets). Therefore, the lowest level of biocontainment should be ventilated cages with negative pressure. In many institutions negative-pressure isolators are considered the only suitable containment devices for housing animals infected with potential human pathogens. Special care is necessary to avoid infections during necropsy of infected animals. Necropsies as well as harvesting tissues or fluids from infected animals should therefore be carried out in safety cabinets.

Like for work with BL3 materials, access to an ABSL-3 facility is highly restricted. Laboratory personnel receive appropriate immunizations (e.g. hepatitis B vaccine). Physical containment devices are necessary for all procedures and manipulations. Animals must be housed in a containment caging system. Individually ventilated cages might be acceptable in specific cases, but negative-pressure isolators or class II biological safety cabinets offer a maximum of safety because supply and removal of infected materials is done in closed containers, thus reliably avoiding a risk of transmission. Very few facilities house ABSL-3 animals. If this is really necessary, much greater safety precautions will be taken than recommended by CDC/NIH (1993) (e.g. one-piece positive-pressure suit ventilated with a life-support system).

ABSL-4 is extremely uncommon and will be avoided whenever possible because transmission of extremely pathogenic organisms to humans is always possible by scratching or biting. Maximum access control and hygiene measures are necessary.

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