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.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.
PART II

Murine Models

I Management of Immunocompromised and Infected Animals

2 In Vitro Analysis
   2.1 Isolation and preparation of lymphocytes from infected animals
   2.2 Establishment of murine T-cell lines and clones, hybridomas and transfecotmas
   2.3 Killer cell assays
   2.4 Measuring cytokine responses by ELISA, ELISPOT and RT-PCR method
   2.5 Isolation of and measuring the function of professional phagocytes: Murine macrophages

3 In Vivo Analysis
   3.1 The immune response in mice challenged with bacterial infections
   3.2 Measuring immune responses

4 Specific Models
   4.1 Murine model of tuberculosis
   4.2 The leishmaniasis model

5 Art and Science of DNA Vaccines

6 Preparation and Use of Adjuvants
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
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 infections.

**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 contami- 
nate viruses, bacteria or parasites that are passaged in laboratory ani-
mals. Spontaneous infections may lead to false conclusions. For example, 
the first isolations of Sendai virus were made from mice that had been inoc-
ulated 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), Guberski et al. (1991) |
| 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         | Schuster et al. (1991)                        |
| *in vitro*                                               |                                               |
| 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 microorganisms 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
Animals 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 (≥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 multi-purpose 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.
- Spirochonulus muris
- Coccidia
- Giardia sp.
- Trichomonads
- Amoebae
- Demodex sp.
- Myobia musculi
- Myocoptes musculus
- Notoedres sp.
- Polyploax 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 microorganisms 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 muriis. 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<sup>csd</sup>, Hfh11<sup>tm</sup>). 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<sup>csd</sup>, 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 x C57BL/6 or 129/Sv x C57BL/6 x 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<sup>tm1H0</sup>) on the original 129/Ola x 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/HeJCr-IIL2<sup>tm1H0</sup> death occurs by 7 weeks and in C57BL/6J-IL2<sup>tm1H0</sup> by 12–24 weeks of age (Mährer 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 (HffI11""), severe combined immunodeficiency (Prkd""), beige (Lyst""), and X-linked immunodeficiency (Btk""). 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 over-expressing 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*

| Locus     | Name                                 | Chromosome | Autoimmunity | None – immune | Special breeding | Special husbandry |
|-----------|--------------------------------------|------------|--------------|---------------|-----------------|------------------|
| bg (Lyst<sup>−</sup>) | Beige                                | 13         |              |              |                 |                  |
| Dh        | Dominant hemimelia                   | 1          |              |              |                 |                  |
| gl        | Grey–lethal                          | 10         |              |              |                 |                  |
| gld (Fasl<sup>−</sup>) | Generalized lymphoproliferative disease | 1         |              |              |                 |                  |
| Hc        | C’5 deficiency                       | 2          |              |              |                 |                  |
| hr        | Hairless                             | 14         |              |              |                 |                  |
| lpr (Fas<sup>+</sup>) | Lymphoproliferation                   | 19         |              |              |                 |                  |
| lps       | Defective lipopolysaccharide response | 4          |              |              |                 |                  |
| me (Heph<sup>−</sup>) | Motheaten                            | 6          |              |              |                 |                  |
| nu (Hfh11<sup>−</sup>) | Nude                                | 11         |              |              |                 |                  |
| oc        | Osteosclerosis                        | 19         |              |              |                 |                  |
| op (Csfl<sup>-</sup>) | Osteopetrosis                       | 3          |              |              |                 |                  |
| scid (Prkdc<sup>−</sup>) | Severe combined immunodeficiency     | 16         |              |              |                 |                  |
| W (Kit<sup>−</sup>) | Dominant spotting                    | 5          |              |              |                 |                  |
| xid (Btk<sup>−</sup>) | 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*

| Locus       | Mutation       | Chromosome | Autoimmunity | None – immune | Special breeding | Special husbandry | Ref.                  |
|-------------|----------------|------------|--------------|---------------|-----------------|------------------|---------------------|
| 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         | Nude           | 10         | –            | +             | +               | +                | Hedrich (1990)       |
| (Hfh11"<sup>nu</sup>; whn") | Toothless | ?          | –            | +             | +               | +                | Hedrich (1990)       |
| il          |                |            |              |               |                 |                  |                     |
| Guinea-pig  |                |            |              |               |                 |                  |                     |
| C2          | C2 deficiency  | Mhc-linked | (+)          | –             | –               | –                | Bitter-Suermann et al. (1981) |
|             |                |            |              |               |                 |                  | Böttger et al. (1986a) |
| C3          | C3 deficiency  | ?          | –            | –             | –               | +                | Böttger et al. (1985) |
| C4          | C4 deficiency  | ?          | (+)          | –             | –               | –                | Böttger et al. (1986b) |
| Syrian hamster |            |            |              |               |                 |                  |                     |
| C6          | C6 deficiency  | ?          | –            | +             | –               | –                | Yang et al. (1974)   |
| nu          | Nude           | ?          | –            | –             | +               | +                | Loridon-Rosa et al. (1988) |

*See footnote to Table 4.
the genes. Prkdc<sup>−/−</sup>, Rag1<sup>+</sup> and Rag2<sup>−/−</sup> 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 immuno-deficiencies. On the other hand, silencing of exon 3 of the whn gene has produced exactly the same phenotype as in Hfh11<sup>−/−</sup> 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
| Mutation          | Chromosome | Autoimmunity | None – immune | Special breeding | Special husbandry | Care                      | Ref.                                      |
|-------------------|------------|--------------|---------------|-----------------|------------------|------------------------|------------------------------------------|
| β2m               | 2          | –            | –             | –               | –                | –                      | Koller et al. (1990)                      |
| Btk               | –          | –            | –             | –               | –                | –                      | Khan et al. (1995)                       |
| Cd4               | 6          | –            | –             | –               | –                | –                      | Locksley et al. (1993)                   |
| Hfh11<sup>–/–</sup> (whn) | 11         | +            | +             | +               | +                | +                      | Nehls et al. (1996)                      |
| Tcrα              | 14         | –            | –             | –               | –                | +                      | Mombaerts et al. (1992a)                 |
| Tcrβ              | 6          | –            | +             | –               | +                | –                      | Mombaerts et al. (1992a)                 |
| Icam1             | 9          | –            | –             | –               | (+)              | –                      | King et al. (1995); Slijkh et al. (1993) |
|                   |            |              |               |                 |                  |                         | Xu et al. (1994)                         |
|                   |            |              |               |                 |                  |                         | Mähler et al. (1996); Sadlack et al. (1993) |
|                   |            |              |               |                 |                  |                         | Kühn et al. (1991); Metwal et al. (1996) |
|                   |            |              |               |                 |                  |                         | Noben-Trauth et al. (1996)               |
| IL2               | 3          | –            | +             | –               | (+)              | –                      | Kopf et al. (1995b)                      |
| IL4               | 11         | –            | +             | –               | (+)              | –                      | Gazzinelli et al. (1996); Kühn et al. (1993) |
| IL6               | 5          | –            | –             | –               | (+)              | –                      | Mattner et al. (1996)                    |
| IL10              | 1          | –            | +             | –               | +                | –                      | Mombaerts et al. (1992)                 |
| IL12              | 6          | –            | –             | –               | (+)              | –                      | Bullard et al. (1996); Mayadas et al. (1993) |
| Rag1/Rag2         | 2          | –            | –             | –               | +                | (+)                    | van Kaer et al. (1992)                   |
| Selp              | 1          | –            | –             | –               | (+)              | –                      | –                                        |
| Tap1              | 17         | –            | –             | –               | (+)              | –                      | –                                        |
| 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 immuno-deficient 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 peracetic 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 \( \text{ft s}^{-1} \) (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 pre-filters.

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 (Trexl, 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 rederviation. 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% CO₂, 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.)
variants of the mouse t complex (T/tw') (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 backcrossed 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).](image-url)
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 harder, 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 (H/f11^{rsu}, H/f11^{rsu-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 backcross 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 Mg²⁺ 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 ³²P 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
   - H₂O 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
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).

(continues)
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 H₂O bidest.
- Staining: 20–30 min, 0.1% AgNO₃ (w/v), 0.037% formaldehyde. (The time of staining depends on the gel concentration and gel thickness.) Rinse for 5–10 s with H₂O bidest.
- Developing: 2–15 min in 2.5% Na₂CO₃; 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
H2D 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_{\text{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_{\text{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.Ba or RT1.Bb allele is identical.

1. Isolate total RNA from tissue.
2. Differentiate the RT1.Ba and RT1.Bb genes by RT-PCR using gene-specific primer pairs.
3. The optimal annealing temperature \((T_{\text{a}})\) 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.Ba and RT1.Bb alleles by reverse dot blot hybridization using two sets of specific oligonucleotides for the RT1.Ba and RT1.Bb alleles (Fig. 4).
7. The oligonucleotides should correspond to the hypervariable areas of exon 2 of the RT1.Ba and RT1.Bb 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.Ba or RT1.Bb allele can be identified by its hybridization pattern (Fig. 4).

![Diagram](image)

**Figure 2.** MHC class I mRNA. \(P_{FW}\), forward primer; \(P_{BW}\), backward primer; \(S_{hybr}\) oligonucleotide.
Figure 3. Identification of H2D alleles by RT-PCR and hybridization using allele-specific oligonucleotides.

Figure 4. Identification of the RT1.B* (left) and RT1.B 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, 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<sup>ps</sup> 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. H2h<sup>11</sup><sup>tm</sup>, Prkd<sup>cat</sup>, Rag1<sup>tm</sup>, Rag2<sup>tm</sup>) a direct demonstration of isohistogeneity is impossible as these animals are
incapable of mounting an allore cognition 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.
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 isohistogenecity 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).
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

| Animals            | Songsasen et al. (1997) | Sztein et al. (1997) | Dorsch and Hedrich (unpublished) |
|--------------------|-------------------------|----------------------|----------------------------------|
|                    | 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 LN₂ | Transfer to −120°C, then transfer to LN₂ | 5 min on dry ice, then transfer to LN₂ |
| 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 (VSI, 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 PBI (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
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 refushing 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 microorganisms.

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 Kunsty (1981)                        |
| Fenbendazole†                    | Panacur (Hoechst)   | Diet for 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 *Aspiculuris 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           | Amoxicillin†         | For 4 weeks – young mice      | 50 mg kg⁻¹   | Russell et al. (1995)         |
| *(Helicobacter hepaticus)†*         | Amoxicillin†         | For 2 weeks                   | 200 mg l⁻¹   | Foltz et al. (1996)           |
|                                     | Metronidazole        |                               | 138 mg l⁻¹   |                               |
|                                     | Bismuth              |                               | 37 mg l⁻¹    |                               |
| Pneumocystosis/mouse, rat           | Sulphamethoxazole–trimethoprim | For 3 weeks                      | 200 mg–1 g l⁻¹ | H. J. Hedrich (unpublished) |
|                                     | (Borgal; Cotrim K, Ratiopharm) |                               | 40–200 mg l⁻¹ | Rülicke (personal communication) |
| Tyzzer’s disease                    | No antibiotic therapy recommended; rederivation | Special disinfectant requiredº |              |                               |
| *(Clostridium piliforme)*/mouse, rat |                      |                               |              |                               |

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
| BL1          | BL2          | BL3          | BL4          |
|-------------|--------------|--------------|--------------|
| **Viruses** |              |              |              |
| Phages      |              |              |              |
| Tobacco mosaic virus | |              |              |
| **Bacteria**|              |              |              |
| Arthrobacter sp. | |              |              |
| Bacillus subtilis | |              |              |
| Erwinia sp.   |              |              |              |
| Lactobacillus acidophilus | |              |              |
| Micrococcus luteus | |              |              |
| Oligella urethralis | |              |              |
| Pseudomonas fluorescens | |              |              |
| Streptomyces sp. | |              |              |
| **Fungi**    |              |              |              |
| Aspergillus niger | |              |              |
| Alternaria alternata | |              |              |
| Malassezia furfur | |              |              |
| Saccharomyces cerevisiae | |              |              |
| Rhizopus oryzae | |              |              |
| **Parasites**|              |              |              |
| Apathogenic trypanosomas | |              |              |

* 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 1 (BL-1)** applies to the use of characterized microorganisms not known to cause disease in healthy human adults. BL-1 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 1 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 1. Fungi that do not infect healthy humans (even if they have the potential to infect immunocompromised hosts) are classified as level 1 (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 burnetii, 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 microorganisms 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.

References

Barthold, S. W. (1986). Research complications and state of knowledge of rodent coronaviruses. In: Complications of Viral and Mycoplasmal Infections in Rodents to Toxicology Research and Testing (T. E. Hamm, Ed.), pp. 53–89. Hemisphere, Washington, DC.

Benoist, C. and Mathis, D. (1993). Transgenes and knockouts in autoimmunity. Curr. Opin. Immunol. 5, 900–902.

Bergs, V. V. (1969). Rat virus-mediated suppression of leukemia induction by Moloney virus in rats. Cancer Res. 29, 1669–1672.
BG Chemie (1990). Tierlaboratorien. Berufsgenossenschaft der chemischen Industrie, Merkblatt M007, 6/90.
BG Chemie (1991a). Sichere Biotechnologie, Eingruppierung biologischer Agenzien: Viren. Berufsgenossenschaft der chemischen Industrie, Merkblatt B004, 4/91.
BG Chemie (1991b). Sichere Biotechnologie, Eingruppierung biologischer Agenzien: Parasiten. Berufsgenossenschaft der chemischen Industrie, Merkblatt B005, 8/91.
BG Chemie (1991c). Sichere Biotechnologie, Eingruppierung biologischer Agenzien: Pilze. Berufsgenossenschaft der chemischen Industrie, Merkblatt B007, 4/91.
BG Chemie (1992). Sichere Biotechnologie, Eingruppierung biologischer Agenzien: Bakterien. Berufsgenossenschaft der chemischen Industrie, Merkblatt B006, 1/92.

Bhatt, P. N., and Jacoby, R. O. (1987). Mousepox in inbred mice innately resistant or susceptible to lethal infection with ectromelia virus. I. Clinical Responses. Lab. Animal Sci. 37, 11–15.

Bhatt, P. N., Jacoby, R. O., Morse, H. C. and New, A. (Eds) (1986a). Viral and Mycoplasma Infections of Laboratory Rodents: Effects on Biomedical Research. Academic Press, New York.

Bhatt, P. N., Jacoby, R. O. and Barthold, S. W. (1986b). Contamination of transplantable murine tumors with lymphocytic choriomeningitis virus. Lab. Animal Sci. 36, 138–139.

Bitter-Suermann, D., Hoffmann, T., Burger, R. and Hadding, U. (1981). Linkage of total deficiency of the second component (C2) of the complement system and of genetic C2-polymorphism to the major histocompatibility complex of the guinea pig. J. Immunol. 127, 608–612.

Böttger, E. C., Hoffmann, T., Hadding, U. and Bitter-Suermann, D. (1985). Influence of genetically inherited complement deficiencies on humoral immune response in guinea pigs. J. Immunol. 135, 4100–4107.

Böttger, E. C., Hoffmann, T., Hadding, U. and Bitter-Suermann, D. (1986a). Guinea pigs with inherited deficiencies of complement components C2 or C4 have characteristics of immune complex disease. J. Clin. Invest. 78, 689–695.

Böttger, E. C., Metzger, S., Bitter-Suermann, D., Stevenson, G., Kleindienst, S. and Burger, R. (1986b). Impaired humoral immune response in complement C3-deficient guinea pigs: absence of secondary antibody response. Eur. J. Immunol. 16, 1231–1235.

Boorman, G. A., Luster, M. L., Dean, J. H., Campbell, M. L., Laker, L. A., Talley, F. A., Wilson, R. E. and Collins, M. J. (1982). Peritoneal and macrophage alterations caused by naturally occurring mouse hepatitis virus. Am. J. Pathol. 106, 110–117.

Boot, R., van Herck, H. and van der Logt, J. (1996). Mutual viral and bacterial infections after housing rats of various breeders within an experimental unit. Lab. Animals 30, 42–45.

Bowen, G. S., Calisher, C. H., Winkler, W. G., Kraus, A. L., Fowler, E. H., Garmann, R. H., Fraser, D. W. and Hinman, A. R. (1975). Laboratory studies of a lymphocytic choriomeningitis virus outbreak in many and laboratory animals. Am. J. Epidemiol. 102, 233–240.

Brown, D. W., Welsh, R. M. and Like, A. A. (1993). Infection of peripancreatic lymph nodes but not islets precedes Kilham rat virus-induced diabetes in BB/Wor rats. J. Virol. 67, 5873–5878.

Bruhin, H. (Ed.) (1989). Planning and Structure of Animal Facilities for Institutes Performing Animal Experiments, Publication No. 1, 2nd edn. Society for Laboratory Animal Science, Biberach.

Bullard, D. C., Hurley, L. A., Lorenzo, I., Sly, L. M., Beaudet, A. L. and Staite, N. D. (1996). Reduced susceptibility to collagen-induced arthritis in mice deficient in intercellular adhesion molecule-1. J. Immunol. 157, 3153–3158.
Burkhardt, F. (1992). Sicherheit im Laboratorium. In: Mikrobiologische Diagnostik (F. Burkhardt, Ed.). Thieme, Stuttgart.
Campbell, D. A., Manders, E. K., Oehler, J. R., Bonnard, G. D., Oldham, R. K. and Herberman, R. B. (1977a). Inhibition of in vitro lymphoproliferative responses by in vivo passaged rat 13762 mammary adenocarcinoma cells. I. Characteristics of inhibition and evidence for an infectious agent. Cell. Immunol. 33, 364–377.
Campbell, D. A., Staal, S. P., Manders, E. K., Bonnard, G. D., Oldham, R. K., Salzman, L. A. and Herberman, R. B. (1977b). Inhibition of in vitro lymphoproliferative response by in vivo passaged rat 13762 mammary adenocarcinoma cells. II. Evidence that Kilham rat virus is responsible for the inhibitory effect. Cell. Immunol. 33, 378–391.
Carrano, V., Barthold, S. W., Beck, D. L. and Smith, A. L. (1984). Alteration of viral respiratory infections of mice by prior infection with mouse hepatitis virus. Lab. Animal Sci. 34, 573–576.
Carter, K. K., Hietala, S. H., Brooks, D. L. and Baggot, J. D. (1987). Tylosin concentrations in rat serum and lung tissue after administration in drinking water. Lab. Animal Sci. 37(4), 468–470.
Carthew, P. and Gannon, J. (1981). Secondary infection of rat lungs with Pasteurella pneumotropica after Kilham rat virus infection. Lab. Animals 15, 219–221.
CCAC (1980). Guide to the Care and Use of Experimental Animals, Vol. 1. Canadian Council of Animal Care, Ottawa, Ontario.
Centers for Disease Control (1988). Agent Summary Statement for Human Immunodeficiency Virus and Report on Laboratory-acquired Infection with Human Immunodeficiency Virus. Addendum I: Vertebrate Animal Biosafety Criteria, MMWR 37 (Suppl. S-4), pp. 11–15. Department of Health and Human Services, Washington, DC.
Centers for Disease Control/National Institute of Health (1993). Biosafety in Microbiological and Biomedical Laboratories, HHS Publication No. (CDC) 93–8395, 3rd edn. US Department of Health and Human Services, Washington, DC.
Choi, G. C., McQuinn, J. S., Jennings, B. L., Hassett, D. J. and Michaels, S. E. (1994). Effect of population size on humidity and ammonia levels in individually ventilated microisolation rodent caging. Contemp. Topics Lab. Animal Sci. 33, 77–81.
Clough, G., Wallace, J., Gamble, M. R., Merryweather, E. R. and Bailey, E. (1995). A positive, individually ventilated caging system: a local barrier system to protect both animals and personnel. Lab. Animals 29, 139–151.
Coleman, D. V. (1978). Obese and diabetes: two mutant genes causing diabetes – obesity syndromes in mice. Diabetologica 14, 141–148.
Collins, M. J. and Parker, J. C. (1972). Murine virus contamination of leukemia viruses and transplantable tumors. J. Natl Cancer Inst. 49, 1139–1143.
Corning, B. F. and Lipman, N. S. (1991). A comparison of rodent caging systems based on microenvironmental parameters. Lab. Animal Sci. 41, 498–503.
Cray, C. et al. (1993). In vitro and long-term in vivo immune dysfunction after infection of BALB/c mice with mouse hepatitis virus strain A59. Lab. Animal Sci. 43, 169–174.
Darrigrand, A. A., Singh, S. B. and Lang, C. M. (1984). Effects of Kilham rat virus on natural killer cell-mediated cytotoxicity in Brown Norway and Wistar Furth rats. Am. J. Vet. Res. 45, 200–202.
Davisson, M. T. (1996). Controls for targeted mutant stocks. JAX Notes 464, 2.
de Souza, M. S., Smith, A. L. and Bottomly, K. (1991). Infection of BALB/cByJ mice with the JHM strain of mouse hepatitis virus alters in vitro splenic T cell proliferation and cytokine production. Lab. Animal Sci. 41, 99–105.
Dick, E. J., Kittell, C. L., Meyer, H., Farrar, P. L., Ropps, S. L., Esposito, J. J., Buller, R. M. L., Neubauer, H., Kang, Y. H. and McKee, A. E. (1996). Mousepox outbreak in a laboratory mouse colony. *Lab. Animal Sci.* 46, 602–611.

Dillehay, D. L., Lehner, N. D. M. and Huerkamp, M. J. (1990). The effectiveness of a microisolator cage system and sentinel mice for controlling and detecting MHV and Sendai virus infections. *Lab. Animal Sci.* 40, 367–370.

Ellermann, K. E., Richards, C. A., Guberski, D. L., Shek, W. R. and Like, A. A. (1996). Kilham rat triggers T-cell dependent autoimmune diabetes in multiple strains of rats. *Diabetes* 45, 557–562.

Enss, M. L., Wagner, S., Liebler, E., Coenen, M. and Hedrich, H.-J. (1997). Response of the germfree rat colonic mucosa. *J. Exp. Animal Sci.* 38, 58–65.

Fallon, M. T., Benjamin, W. H., Schoeb, T. R. and Briles, D. E. (1991). Mouse hepatitis virus strain UAB infection enhances resistance to *Salmonella typhimurium* in mice by inducing suppression of bacterial growth. *Infect. Immun.* 59, 852–856.

FELASA (1995). Recommendations on the education of persons working with laboratory animals: categories A and C. *Lab. Animals* 29, 121–131.

Festing, F. W. (1979). *Inbred Strains in Biomedical Research.* Macmillan Press London.

Fidler, I. J. (1977). Depression of macrophages in mice drinking hypochlorinated water. *Nature* 270, 735–736.

Foltz, C. J., Fox, J. G., Yan, L. and Shames, B. (1996). Evaluation of various oral antimicrobial formulations for eradication of *Helicobacter hepaticus*. *Lab. Animal Sci.* 46, 193–197.

Fox, J. G., Dewhirst, F. E., Tully, J. G., Paster, B. J., Yan, L., Taylor, N. S., Collins, M. J., Gorelick, P. L. and Ward, J. M. (1994). *Helicobacter hepaticus* sp. nov., a microaerophilic bacterium isolated from livers and intestinal mucosa scrapings from mice. *J. Clin. Microbiol.* 32, 1238–1245.

Fox, J. G., Yan, L., Dewhirst, F. E., Paster, B. J., Shames, B., Murphy, J. C., Hayward, A., Belcher, J. C. and Mendes, E. N. (1995). *Helicobacter bilis* sp. nov., a novel *Helicobacter* species isolated from bile, livers and intestines of aged, inbred mice. *J. Clin. Microbiol.* 33, 445–454.

Gabaldon, M., Capdevila, C. and Zuniga, A. (1992). Effect of spontaneous pathological and thrombin on leukocyte adhesion to rat aortic endothelium. *Atherosclerosis* 93, 217–228.

Gazzinelli, R. T., Wysocka, M., Hieny, S., Scharton-Kersten, T., Cheever, A., Kuhn, R., Muller, W., Trinchieri, G. and Sher, A. (1996). In the absence of endogenous IL-10, mice acutely infected with *Toxoplasma gondii* succumb to a lethal immune response dependent on CD4+ T cells and accompanied by overproduction of IL-12, IFN-γ and TNF-α. *J. Immunol.* 157, 798–805.

Goelz, M. F., Thigpen, J. E., Mahler, J., Rogers, W.P., Locklear, J., Weigler, B. J. and Forsythe, D. B. (1996). Efficacy of various therapeutic regimens in eliminating *Pasteurella pneumotropica* from the mouse. *Lab. Animal Sci.* 46, 280–284.

Green, E. L. (1966). *Biology of the Laboratory Mouse.* McGraw-Hill, New York.

Green, E. L. (1981). *Genetics and Probability in Animal Breeding Experiments.* Macmillan, London.

Gripenberg-Lerche, C. and Toivanen, P. (1993). *Yersinia* associated arthritis in SHR rats: effect of the microbial status of the host. *Ann. Rheum. Dis.* 52, 223–228.
Gripenberg-Lerche, C. and Toivanen, P. (1994). Variability in the induction of experimental arthritis: Yersinia associated arthritis in Lewis rats. Scand. J. Rheumatol. 23, 124–127.

Gröschel, D. H. M. and Strain, B. A. (1991). Laboratory safety in clinical microbiology. In: Manual of Clinical Microbiology (A. Balows, W. J. Hansler, Jr, K. L. Herrmann, H. D. Isenberg and H. J. Shadomy, Eds), 5th edn, pp. 49–58. American Society for Microbiology, Washington, DC.

Gu, H., Kitamura, D. and Rajewsky, K. (1991). B cell development regulated by gene rearrangement: arrest of maturation by membrane-bound Dp protein and selection of Dm element reading frames. Cell 65, 47–54.

Guberski, D. I., Thomas, V. A., Shek, W. R., Like, A. A., Handler, E. S., Rossini, A. A., Wallace, J. E. and Welsh, R. M. (1991). Induction of type I diabetes by Kilham rat virus in diabetes-resistant BB/WOR rats. Science 254, 1010–1013.

Hamm, T. E. (Ed.) (1986). Complications of Viral and Mycoplasmal Infections in Rodents to Toxology Research and Testing. Hemisphere, Washington, DC.

Hansen, A. K. (1994). Health status and the effects of microbial organisms on animal experiments. In: Handbook of Laboratory Animal Science, Vol. 1 (P. Svendsen and J. Hau, Eds), pp. 125–153. CRC Press, Boca Raton, FL.

Hansen, A. K. (1995). Antibiotic treatment of nude rats and its impact on the aerobic bacterial flora. Lab. Animals 29, 37–44.

Hansen, C. T., Judge, F. J. and Whitney, R. A. (1973). Catalogue of NIH Rodents, DHEW Publication No. 74-606. US Department of Health, Education and Welfare, Washington, DC.

Harkness, J. E. and Wagner, J. E. (1983). The Biology and Medicine of Rabbits and Rodents. Lea & Febiger, Philadelphia.

Hasenau, J. J., Baggs, R. B. and Kraus, A. L. (1993). Microenvironments in microisolation cages using BALB/c and CD-1 mice. Contemp. Topics Lab. Animal Sci. 32, 11–16.

Hawk, C. T. and Leary, S. L. (1995). Formulary for Laboratory Animals. Iowa State University Press, Ames, IA.

Hedrich, H. J. (Ed.) (1990). Genetic Monitoring of Inbred Strains of Rats. Gustav Fischer Verlag, Stuttgart.

Hedrich, H. J. and Reetz, I. (1990). Cryopreservation of rat embryos. In: Genetic Monitoring of Inbred Strains of Rats (H.-J. Hedrich, Ed.), pp. 274–288. Gustav Fischer Verlag, Stuttgart.

Heine, W. (1980). How to define SPF? Z. Versuchstierk. 22, 262–266.

Herman, L. M., White, W. J. and Lang, C. M. (1982). Prolonged exposure to acid, chlorine, or tetracycline in the drinking water: effects on delayed-type hypersensitivity, hemagglutination titers, and reticuloendothelial clearance rates in mice. Lab. Animal Sci. 32, 603–608.

Hirsjärvi, P. and Phyälä, L. (1995). Ivermectin treatment of a colony of guinea pigs infested with fur mite (Chirodiscoides caviae). Lab. Animals 29, 200–203.

Hogan, B., Beddington, R., Costantini, F. and Lacy, E. (1994). Manipulating the Mouse Embryo. A Laboratory Manual, pp. 117–182. Cold Spring Harbor Laboratory Press, New York.

Home Office (1989). Code of Practice for the Housing and Care of Animals used in Scientific Procedures, pp. 1–33. HMSO, London.

Huerkamp, M. J. and Lehner, N. O. M. (1994). Comparative effects of forced-air, individual cage ventilation or an absorbent bedding additive on mouse isolator cage microenvironment. Contemp. Topics Lab. Animal Sci. 33, 58–61.

Hunskaar, S. and Fosse, R. T. (1993). Allergy to laboratory mice and rats: a review of its prevention, management, and treatment. Lab. Animals 27, 206–221.
ILAR Committee on Immunologically Compromised Rodents (National Research Council) (1989). *Immunodeficient Rodents. A Guide to their Immunobiology, Husbandry, and Use.* National Academic Press, Washington, DC.

ILAR Committee on Long-term Holding of Laboratory Rodents (1976). Long-term holding of laboratory rodents. *ILAR News* XIX(4), L1–L25.

ILAR Committee Report (National Research Council) (1997). *Occupational Health and Safety in the Care and Use of Research Animals.* National Academic Press, Washington, DC.

Jacoby, R. O., Ball-Goodrich, L. J., Besselsen, D. G., McKisic, M. D., Riley, L. K. and Smith, A. L. (1996). Rodent parvovirus infections. *Lab. Animal Sci.* 46, 370–380.

Kaufmann, S. H. E. and Ladel, C. H. (1994). Application of knockout mice to the experimental analysis of infections with bacteria and protozoa. *Trends Microbiol.* 2, 235–241.

Kawamata, J., Yamanouchi, T., Dohmae, K., Miyamoto, H., Takahashi, M., Yamanishi, K., Kurata, T. and Lee, H. W. (1987). Control of laboratory acquired hemorrhagic fever with renal syndrome (HFRS) in Japan. *Lab. Animal Sci.* 37, 431–436.

Keller, L. S. F., White, W. J., Snider, M. T. and Lang, C. M. (1989). An evaluation of intra-cage ventilation in three animal caging systems. *Lab. Animal Sci.* 39, 237–241.

Khan, W. N., Sideras, P., Rosen, F. S. and Alt, F. W. (1995). The role of Bruton’s tyrosine kinase in B-cell development and function in mice and man. *Ann. NY Acad. Sci.* 764, 27–38.

Kilham, L. and G. Margolis (1966). Spontaneous hepatitis and cerebellar ‘hypoplasia’ in suckling rats due to congenital infections with rat viruses. *Am. J. Pathol.* 49, 457–475.

Kilham, L., Buckler, C. E., Ferm, V. H. and Baron, S. (1968). Production of interferon during rat virus infection. *Proc. Soc. Exp. Biol. Med.* 129, 274–278.

King, P. D., Sandberg, E. T., Selvakumar, A., Fang, P., Beaudet, A. L. and Dupont, B. (1995). Novel isoforms of murine intercellular adhesion molecule-1 generated by alternative RNA splicing. *J. Immunol.* 154, 6080–6093.

Klement, P., Augustine, J. M., Delaney, K. H., Klement, G. and Weitz, J. I. (1996). An oral ivermectin regimen that eradicates pinworms (*Syphacia* spp.) in laboratory rats and mice. *Lab. Animal Sci.* 46, 286–290.

Koller, B. H., Marrack, P., Kappler, J. W. and Smithies, O. (1990). Normal development of mice deficient in β-2m MHC class I proteins, and CD8+ T cells. *Science* 248, 1227–1229.

Koopman, J. P., van den Brink, M. E., Vennix, P. P. C. A., Kuypers, W., Boot, R. and Bakker, R. H. (1991). Isolation of *Streptobacillus moniliformis* from the middle ear of rats. *Lab. Animals* 25, 35–39.

Kopf, M., Le Gros, G., Coyle, A. J., Kosco-Vilbois, M. and Brombacher, F. (1995a). Immune responses of IL-4, IL-5, IL-6 deficient mice. *Immunol. Rev.* 148, 45–69.

Kopf, M., Ramsay, A., Brombacher, F., Baumann, H., Freer, G., Galanos, C., Gutierrez-Ramos, J. C. and Kohler, G. (1995b). Pleiotropic defects of IL-6 deficient mice including early hematopoiesis, T and B cell function, and acute phase responses. *Ann. NY Acad. Sci.* 762, 308–318.

Kraft, L. M. (1958). Observations on the control and natural history of epidemic diarrhea of infant mice (EDIM). *Yale J. Biol. Med.* 31, 121–137.

Kraft, V., Deeny, A. A., Blanchet, H. M., Boot, R., Hansen, A. K., Hem, A., van Herck, H., Kunsty, L., Milte, G., Needham, J. R., Nicklas, W., Perrot, A., Rehbinder, C. and Richard, Y. (1994). Recommendations for the health monitoring of mouse, rat, hamster, guineapig and rabbit breeding colonies. Report of
Kühn, R., Rajewsky, K. and Müller, W. (1991). Generation and analysis of interleukin-4-deficient mice. Science 254, 707-710.
Kühn, R., Löhler, J., Rennick, J., Rajewsky, K. and Müller, W. (1993). Interleukin-10-deficient mice develop chronic enterocolitis. Cell 75, 263-274.
Kuhner, M. K. and Goodenow, R. S. (1989). Mouse H-2 and Qa sequences. Immunogenetics 30, 458-464.
Kunstyr, I. (Ed.) (1988a). List of Pathogens for Specification in SPF Laboratory Animals, Publication No. 2. Society for Laboratory Animal Science, Biberach.
Kunstyr, I. (Ed.) (1988b). Hygiene-Empfehlungen für Versuchstierbereiche, Publication No. 5/6. Society for Laboratory Animal Science, Biberach.
Kunstyr, I. (Ed.) (1992). Diagnostic microbiology for laboratory animals. In: GV-SOLAS, Vol. 11. Gustav Fischer Verlag, Stuttgart.
Kyriazis, A. P., DiPersio, L., Michael, J. G. and Pesce, A. J. (1979). Influence of the mouse hepatitis virus (MHV) infection on the growth of human tumors in the athymic mouse. Int. J. Cancer 23, 402-409.
Lane-Petter, W. and Pearson, A. E. G. (1971). The Laboratory Animal – Principles and Practice. Academic Press, London.
Le Blanc, S., Faith, R. E. and Montgomery, C. A. (1993). Use of topical ivermectin treatment for Syphacia obvelata in mice. Lab. Animal Sci. 43, 526-528.
Lee, A., Phillips, M. W., O'Rourke, J. L., Paster B. J., Dewhirst, F. E., Fraser, G. J., Fox, J. G., Sly, L. I., Romaniuk, P. J., Trust, T. J. and Koupach, S. (1992). Helicobacter muridarum sp. nov., a microaerophilic helical bacterium with a novel ultrastructure isolated from the intestinal mucosa of rodents. Int. J. Syst. Bacteriol. 42, 27-36.
Leibo, S. P. (1981). Introduction to embryo freezing. In: Frozen Storage of Laboratory Animals (G. H. Zeilmaker, Ed.), pp. 1-19. Gustav Fischer Verlag, Stuttgart.
Leiter, E. H., Coleman, D. L. and Eppig, I. I. (1979). Endocrine pancreatic cells of postnatal 'diabetes' (db) mice in cell culture. In Vitro 15, 507-521.
Levy, G. A., Leibowitz, J. L. and Edgington, T. S. (1981). Induction of monocyte procoagulant activity by murine hepatitis virus type 3 parallels disease susceptibility in mice. J. Exp. Med. 154, 1150-1163.
Lewis, D. B., Liggitt, H. D., Effmann, E. L., Motley, S. T., Teilbaum, S. L., Jespen, K. J., Goldstein, S. A., Bonadio, J., Carpenter, J. and Perlmutter, R. M. (1993). Osteoporosis induced in mice by overproduction of interleukin 4. Proc. Natl Acad. Sci. USA 90, 11 618-11 622.
Lipman, N. S. (1992). Microenvironmental conditions in isolator cages: an important research variable. Lab. Animals 21, 23-26.
Lipman, N. S. (1993). Strategies for architectural integration of ventilated caging systems. Contemp. Topics Lab. Animal Sci. 32, 7-10.
Lipman, N. S., Corning, B. F. and Coiro, M. A. (1992). The effects of intracage ventilation on microenvironmental conditions in filter-top cages. Lab. Animals 26, 206-210.
Lipman, N. S., Corning, B. F. and Saifuddin, M. (1993). Evaluation of isolator caging systems for protection of mice against challenge with mouse hepatitis virus. Lab. Animals 27, 134-140.
Lipman, N. S., Dalton, S. D., Stuart, A. R. and Arruda, K. (1994). Eradication of Pinworms (Syphacia obvelata) from a large mouse breeding colony by combination oral anthelmitic therapy. Lab. Animal Sci. 44, 517-520.
Locksley, R. M., Reiner, S. L., Hatam, F., Littman, D. R. and Killeen, N. (1993). Helper T cells without CD4: control of leishmaniasis in CD4-deficient mice. *Science* **261**, 1448–1451.

Loridon-Rosa, B., Haddada, H. and de Vaux St Cyr, C. (1988). The nude hamster: a potential new animal model for experimental cancer studies. *Cancer J.* **2**, 112.

Lucchiarl, M. A., Pereira, C. A., Kuhn, L. and Lefkovits, I. (1992). The pattern of proteins synthesized in the liver is profoundly modified upon infection of susceptible mice with mouse hepatitis virus 3. *Res. Virol.* **143**, 231–240.

Lussier, G. (1988). Potential detrimental effects of rotoviral infections on long-term experiments. *Vet. Res. Contrib.* **12**, 199–217.

Lyon, M. F., Searle, A. G., Ford, G. E. and Ohno, S. (1964). A mouse translocation suppressing sex-linked variegation. *Cytogenetics* **3**, 306–323.

Lyon, M. F., Rastan, S. and Brown, S. D. M. (Eds) (1996). *Genetic Variants and Strains of the Laboratory Mouse*, 3rd edn. Oxford University Press, Oxford.

Mähler, M., Serreze, D., Evans, R., Cutler Linder, C., Leiter, E. and Sundberg, J. P. (1996). II.\textsuperscript{aanti} an interleukin-2 gene targeted mutation. *Jax Notes* **467**, 2–4.

Maess, J. and Kunstyr, I. (1981). Diagnose und Bekämpfung häufiger Parasiten bei kleinen Versuchstieren. *Tierärztl. Prax.* **9**, 259–264.

Manaker, M., Riczak, C. V., Miller, A. A. and Stanton, M. F. (1961). A hepatitis virus complicating studies with mouse leukemia. *J. Natl Cancer Inst.* **27**, 29–51.

Margolis, G. and Kilham, L. (1975). Problems of human concern arising from animal models of intrauterine and neonatal infections due to viruses: a review. II. Pathologic studies. *Prog. Med. Virol.* **20**, 144–179.

Mattner, F., Magram, J., Ferrante, J., Launois, P., Di Padova, K., Behin, R., Gately, M. K., Louis, J. A. and Alber, G. (1996). Genetically resistant mice lacking interleukin-12 are susceptible to infection with *Leishmania major* and mount a polarized Th2 cell response. *Eur. J. Immunol.* **26**, 1553–1559.

Mayadas, T. N., Johnson, R. C., Rayburn, H., Hynes, R. O. and Wagner, D. D. (1993). Leukocyte rolling and extravasation are severely compromised in P-selectin-deficient mice. *Cell* **74**, 541–554.

McKisic, M. D., Faturzo, F. X., Gaertner, D. J., Jacoby, R. O. and Smith, A. L. (1995). A nonlethal parvovirus infection suppresses rat T lymphocyte effector functions. *J. Immunol.* **155**, 3979–3986.

Mendes da Cruz, G. (1991). Der Einfluss des Einfrierverfahrens auf die Revitalisierungsrate von Mäuseembryonen. Diss. Tierärztl. Hochschule, Hannover.

Metwali, A., Elliott, D., Blum, A. M., Li, J., Sandor, M., Lynch, R., Noben-Trauth, N. and Weinstock, J. V. (1996). The granulomatous response in murine *Schistosomiasis mansoni* does not switch to Th1 in IL-4-deficient C57BL/6 mice. *J. Immunol.* **157**, 4546–4553.

Miller, S. A., Dykes, D. D. and Polenky, H. F. (1989). A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acid Res.* **16**, 1215.

Mittreuecker, H.-W., Pfeffer, K., Schmits, R. and Mak, M. W. (1995). T-lymphocyte development and function in gene-targeted mutant mice. *Immunol. Rev.* **148**, 115–150.

Mombaerts, P., Clarke, A. R., Rudnicki, M. A., Lacomini, J., Itohara, S., Lafaille, J. J., Wang, L., Ichikawa, Y., Jaenisch, R., Hooper, M. L. and Tonegawa, S. (1992a). Mutations in T-cell antigen receptor genes α and β block thymocyte development at different stages. *Nature* **360**, 225–231.

Mombaerts, P., Lacomini, J., Johnson, R. S., Herrup, K., Tonegawa, S. and Papaioannou, V. E. (1992b). RAG-1-Deficient mice have no mature B and T lymphocytes. *Cell* **68**, 869–877.
Tonegawa, S. (1993). Spontaneous development of inflammatory bowel disease in T cell receptor mutant mice. *Cell* 75, 275–282.

Morris, R. H. (1995). Antibiotic therapeutics in laboratory animals. *Lab. Animals* 29, 16–36.

Mossmann, H. (1992). The SCID mouse: description, husbandry and experimental use. *Contrib. Oncol.* 42, 287–293.

Nakagata, N. and Takeshima, T. (1992). High fertilization ability of mouse spermatozoa diluted slowly after cryopreservation. *Theriogenology* 37, 1283–1291.

National Research Council, Committee on Infectious Diseases of Mice and Rats (1991). *Infectious Diseases of Mice and Rats*. National Academy Press, Washington, DC.

National Research Council (1996). *Guide for the Care and Use of Laboratory Animals*. National Academy Press, Washington, DC.

Nehls, M., Kyewski, B., Messerle, M., Waldschutz, R., Schüddekopf, K., Smith, A. I. and Boehm, T. (1996). Two genetically separable steps in the differentiation of thymic epithelium. *Science* 272, 886–889.

Nicklas, W. (1993). Possible routes of contamination of laboratory rodents kept in research facilities. *Scand. J. Lab. Animal Sci.* 20, 53–60.

Nicklas, W. (1996). Health monitoring of experimental rodent colonies; an overview. *Scand. J. Lab. Animal Sci.* 23, 69–75.

Nicklas, W., Giese, M., Zawatzky, R., Kirchner, H. and Eaton, P. (1988). Contamination of a monoclonal antibody with LDH-virus causes interferon induction. *Lab. Animal Sci.* 38, 152–154.

Nicklas, W., Kraft, V. and Meyer, B. (1993a). Contamination of transplantable tumors, cell lines, and monoclonal antibodies with rodent viruses. *Lab. Animal Sci.* 43, 296–300.

Nicklas, W., Staut, M. and Benner, A. (1993b). Prevalence and biochemical properties of V factor-dependent Pasteurellaceae from rodents. *Zentralbl. Bakt.* 279, 114–124.

Noben-Trauth, N., Kropf, P. and Muller, I. (1996). Susceptibility to *Leishmania major* infection in interleukin-4-deficient mice. *Science* 271, 987–990.

O'Rourke, J., Lee, A. and McNeill, J. (1988). Differences in the gastrointestinal microbiota of specific pathogen-free mice: an often unknown variable in biomedical research. *Lab. Animals* 22, 297–303.

Oshugi, T., Kiuchi, Y., Shimoda, K., Oguri, S. and Maejima, K. (1996). Translocation of bacteria from the gastrointestinal tract in immunodeficient mice. *Lab. Animals* 30, 46–50.

Otis, A. P. and Foster, H. L. (1983). Management and design of breeding facilities. In: *The Mouse in Biomedical Research*, Vol. 3 (H. L. Foster, J. D. Small and J. G. Fox, Eds), pp. 18–35. Academic Press, Orlando, FL.

Otsen, M. (1995). Use of DNA markers for genetic characterization and linkage studies in the rat (*Rattus norvegicus*). Proeefschrift Universiteit Utrecht.

Peltier, A. P. (1982). Complement, In: *Immunology* (J.-F. Bach, Ed.), 2nd edn, pp. 252–284. Wiley, New York.

Perkins, S. E. and Lipman, N. S. (1996). Evaluation of microenvironmental conditions and noise generation in three individually ventilated rodent caging systems and static isolator cages. *Contemp. Topics Lab. Animal Sci.* 35, 61–65.

Piazza, M., Piccinio, F. and Matano, F. (1965). Hematological changes in viral (MHB-3) murine hepatitis. *Nature* 205, 1034–1035.

Rall, W. F. (1981). The role of intracellular ice in the slow warming injury of mouse embryos. In: *Frozen Storage of Laboratory Animals* (G. H. Zeilmaker, Ed.), pp. 33–44. Gustav Fischer Verlag, Stuttgart.
Rall, W. F. and Fahy, G. M. (1985). Ice-free cryopreservation of mouse embryos at
−196°C by vitrification. Nature 313, 573–575.
Rall, W. F., Reid, D. S. and Farrant, J. (1980). Innocuous biological freezing during
warming. Nature 286, 511–514.
Reetz, I. C., Wullenweber-Schmidt, M., Kraft, V. and Hedrich, H.-J. (1988).
Rederivation of inbred strains of mice by means of embryo transfer. Lab. Animal
Sci. 38, 696–701.
Rehbinder, C., Baneux, P., Forbes, D., van Herck, H., Nicklas, W., Rugaya, Z. and
Winkler, G. (1996). Recommendations for the health monitoring of mouse, rat,
hamster, gerbil, guineapig and rabbit experimental units. Report of the
Federation of European Laboratory Animal Science Associations (FELASA)
Working Group on Animal Health. Lab. Animals 30, 193–208.
Renard, J. P. and Babinet, C. (1984). High survival of mouse embryos after rapid
freezing and thawing inside plastic straws with 1,2-propanediol as cryo-
protectant. J. Exp. Zool. 230, 443–448.
Riley, V. (1966). Spontaneous mammary tumors: decrease of incidence in mice
infected with an enzyme-elevated virus. Science 153, 1657–1658.
Rodrigue, L. and Lavoie, M. C. (1996). Comparison of the proportions of oral
bacterial species in BALB/c mice from different suppliers. Lab. Animals 30,
108–113.
Rouleau, A. M. J., Kovacs, P. R., Kunz, H. W. and Armstrong, D. T. (1993).
Decontamination of rat embryos and transfer to specific pathogen-free
recipients for the production of a breeding colony. Lab. Anim. Sci. 43,
611–617.
Rozengurt, N. and Sanchez, S. (1993). A spontaneous outbreak of Theiler’s
encephalomyelitis in a colony of severe combined immunodeficient mice in the
UK. Lab. Animals 27, 229–234.
Russell, R. J., Haines, D. C., Anver, M. R., Battles, J. K., Gorelick, P. L., Blumenauer,
L. L., Gonda, M. A. and Ward, J. M. (1995). Use of antibiotics to prevent hepatitis
and typhlitis in male SCID mice spontaneously infected with Helioptacter
hepaticus. Lab. Animal Sci. 45, 373–378.
Rychlik, W. and Roads, R. E. (1989). A computer program for choosing optimal
oligonucleotides for filter hybridization, sequencing and in vitro amplification
of DNA. Nucleic Acid. Res. 17, 8543–8551.
Rychlik, W., Spencer, W. J. and Rhoads, R. E. (1990). Optimization of the annael-
ting temperature for DNA amplification in vitro. Nucleic Acid. Res. 18, 6409–6412.
Sadlack, B., Merz, H., Schorle, H., Schimpl, A., Feller, A. C. and Horak, I. (1993).
Ulcerative colitis-like disease in mice with a disrupted interleukin-2 gene. Cell
75, 253–261.
Sambrook, J., Fritsch, E. F. and Maniatis, T. (Eds) (1989). Molecular Cloning. A
Laboratory Manual, 2nd edn. Cold Spring Harbor Laboratory Press, New
York.
Schauer, D. B., Zabel, B. A., Pedraza, I. F., O’Hara, C. M., Steigerwalt, A. G. and
Brenner, D. J. (1995). Genetic and biochemical characterization of Citrobacter
rodentium sp. nov. J. Clin. Microbiol. 33, 2064–2068.
Schenkel, J. (1995). Transgene Tiere, pp. 61–108. Spektrum Akademischer Verlag,
Heidelberg.
Schindler, L., Engler, H. and Kirchner, H. (1982). Activation of natural killer cells
and induction of interferon after infection of mouse hepatitis virus type 3 in
mice. Infect. Immun. 35, 869–873.
Schiewe, M. C., Schmidt, P. M., Bush, M. and Wildt, D. E. (1985). Toxicity potential
of absorbed-retained ethylene oxide residues in culture dishes on embryo
development in vitro. Lab. Animal Sci. 60, 1610–1618.

183
Schoeb, T. R., Davidson, M. K. and Lindsey, J. R. (1982). Intracage ammonia promotes growth of Mycoplasma pulmonis in the respiratory tract of rats. Infect. Immun. 38, 212–217.

Schuster, G. S., Caughman, G. B. and O’Dell, N. L. (1991). Altered lipid metabolism in parvovirus-infected cells. Microbios 66, 134–155.

Scopets, B., Wilson, R. P., Griffith, J. W. and Lang, C. M. (1996). Ivermectin toxicity in young mice. Lab. Animal Sci. 46, 111–112.

Scott, R. W., Sudweeks, J. and Teuscher, C. (1992). Random sequence oligonucleotide primers detect polymorphic DNA products which segregate in inbred strains of mice. Mammalian Genome 3, 73–78.

Serrano, L. J. (1971). Carbon dioxide and ammonia in mouse cages: effect of cage cover, population, and activity. Lab. Animal Sci. 21, 75–85.

Sherwin, C. M. (1997). Observation on the prevalence of nest-building in non-breeding TO strain mice and their use of two nesting materials. Lab. Animals 31, 125–132.

Sibilia, M. and Wagner, E. F. (1995). Strain-dependent epithelial defects in mice lacking the EGF receptor. Science 269(5221), 234–238.

Silver, L. M. (1985). Mouse t haplotypes. Ann. Rev. Genet. 19, 179–208.

Sligh, J. E., Ballantyne, C. M., Rich, S. S., Hawkins, H. K., Smith, C. W., Bradley, A. and Beaudet, A. L. (1993). Inflammatory and immune responses are impaired in mice deficient in intercellular adhesion molecule 1. Proc. Natl Acad. Sci. USA 90, 8529–8533.

Smith, A. L., Casals, J. and Main, A. J. (1983). Antigenic characterization of tettnang virus: complications caused by passage of the virus in mice from a colony enzootically infected with mouse hepatitis virus. Am. J. Trop. Med. Hyg. 32, 1172–1176.

Songsasen, N., Betteridge, K. J. and Leibo, S. P. (1997). Birth of live mice resulting from oocytes fertilized in vitro with cryopreserved spermatozoa. Biol. Reprod. 56, 143–152.

Spencer, H. J. (1967). Recovery of rat virus from a series of chemically induced rat leukemias. Am. Assoc. Cancer Res. 8, 62.

Spiegel, A. (1976). Versuchstiere: Eine Einführung in die Grundlagen ihrer Zucht und Haltung. Gustav Fischer Verlag, Stuttgart.

Stevens, L. C. (1957). A modification of Robertson’s technique of homoiotopic ovarian transplantation in mice. Transplant Bull. 4, 106–107.

Strasser, A., Whittingham, S., Vaux, D. L., Bath, M. L., Adams, J. M., Cory, S. and Harries, A. W. (1991). Enforced BCL2 expression in B-lymphoid cells prolongs antibody responses and elicits autoimmune disease. Proc. Natl Acad. Sci. USA 88, 8861–8665.

Strasser, H. and Tiefenbach, B. (1977). Erfahrungen mit Fenbendazol bei der Bekämpfung von Syphacia muris in einer Rattenzucht. Dtsch. Tierärztl. Wschr. 84, 479–480.

Sztein, J. M., Farley, J. S., Young, A. F. and Mobraaten, L. E. (1997). Motility of cryopreserved mouse spermatozoa affected by temperature of collection and rate of thawing. Cryobiology 35, 46–52.

Thigpen, J. E. and Ross, P. W. (1983). Viral cross contamination of rats maintained in a fabric-walled mass air flow system. Lab. Animal Sci. 33, 446–450.

Tietjen, R. M. (1992). Transmission of minute virus of mice into a rodent colony by a research technician. Lab. Animal Sci. 42, 422.

Trexler, P. C. (1983). Gnotobiotics. In: The Mouse in Biomedical Research (H. L. Foster, J. D. Small and J. G. Fox, Eds), pp. 1–16. Academic Press, Orlando, Fl.

Trinchieri, G. (1997). Cytokines acting on or secreted by macrophages during intracellular infection (Il-10, Il-12, IFN-γ). Curr. Opin. Immunol. 9, 17–23.
Tu, H., Diberadinis, L. J. and Lipman, N. S. (1997). Determination of air distribution, exchange, velocity, and leakage in three individually ventilated rodent caging systems. *Contemp. Topics Lab. Animal Sci.* 36, 69–73.

Van de Weerd, H. A., Van Loo, P. L. P., Van Zutphen, L. F. M., Koolhaas, J. M. and Baumans, V. (1997). Preferences for nesting material as environmental enrichment for laboratory mice. *Lab. Animals* 31, 133–143.

van Kaer, L., Ashton-Rickardt, P. G., Ploegh, H. L. and Tonegawa, S. (1992). TAP1 mutant mice are deficient in antigen presentation, surface class I molecules, and CD4+ T cells. *Cell* 71, 1205–1214.

Viney, J. L. (1994). Transgenic and knockout models for studying diseases of the immune system. *Curr. Opin. Genet. Dev.* 4, 461–465.

Virelizier, J. L., Virelizier, A. M. and Allison, A. C. (1976). The role of circulating interferon in the modifications of the immune responsiveness by mouse hepatitis virus (MHV-3). *J. Immunol.* 117, 748–753.

Waggie, K., Kagiyami, N., Allen, A. M. and Nomura, T. (Eds) (1994). *Manual of Microbiologic Monitoring of Laboratory Animals*, 2nd edition, NIH Publication No. 94-2498. US Department of Human Health and Human Services, Washington, DC.

Wakeland, E. K., Boehme, S. and She, J. X. (1990). The generation and maintenance of MHC class II gene polymorphism in rodents. *Immunol. Rev.* 113, 207–226.

Wakeland, E. K., Morel, L., Achey, K., Yui, M. and Longmate, J. (1997). Speed congenics: a classic technique in the fast lane (relatively speaking). *Immunol. Today* 18, 472–477.

Weihe, W. H. (1978). Richtwerte der Belegungsdichte von Standardgefäßen für kleine Versuchstiere. *Z. Versuchstierk.* 20, 305–309.

Weisbroth, S. H. (1982). Arthropods. In: *The Mouse in Biomedical Research*, Vol. II (H. L. Foster, J. O. Small and J. G. Fox, Eds), pp. 385–402. Academic Press, Orlando, FL.

Weisbroth, S. H. (1996). Post-indigenous disease: changing concepts of disease in laboratory rodents. *Lab. Animals* 25(9), 25–33.

Whitten, W. K. (1971). Embryo medium. Nutrient requirements for the culture of preimplantation embryos in vitro. *Adv. Biosci.* 6, 129–141.

Whittingham, D. G. (1971). Culture of mouse ova. *J. Reprod. Fertil.* 14, 7–21.

Whittingham, D. G., Leibo, S. P. and Mazur, P. (1972). Survival of mouse embryos frozen to -196°C and -269°C. *Science* 78, 411–414.

Wilbertz, S., Partke, H. J., Dagnaes-Hansen, F. and Herberg, L. (1991). Persistent MHV (mouse hepatitis) infection reduces the incidence of diabetes mellitus in non-obese diabetic mice. *Diabetologia* 34, 2–5.

Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A. and Tingey, S. V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acid. Res.* 18 (22), 6531–6535.

Wilmut, I. (1972). The effect of cooling rate, warming rate, cryoprotective agent and stage of development on survival of mouse embryos during freezing and thawing. *Life Sci.* 11, 1072–1079.

Wollnik, F. (1989). Physiology and regulation of biological rhythms in laboratory animals: an overview. *Lab. Animals* 23, 107–125.

Wozniak, J. and Hetrick, F. (1969). Persistent infection of a rat nephroma cell line with Kilham rat virus. *J. Virol.* 4, 313–314.

Wullenweber, M., Kaspareit-Rittinghausen, J. and Farouq, M. (1990). *Streptobacillus moniliformis* epizootic in barrier-maintained C57BL/6 mice and susceptibility to infection of different strains of mice. *Lab. Animal Sci.* 40, 608–612.
Xu, H., Gonzalo, J. A., St. Pierre, Y., Williams, I. R., Kupper, T. S., Cotran, R. S., Springer, T. A. and Gutierrez-Ramos, J.-C. (1994). Leukocytosis and resistance to septic shock in intercellular adhesion molecule 1-deficient mice. J. Exp. Med. 180, 95–109.

Yamanishi, K., Dantas, J. R. F., Takahashi, M., Yamanouchi, T., Domae, K., Kawamata, J. and Kurata, T. (1983). Isolation of hemorrhagic fever with renal syndrome (HFRS) virus from a tumor specimen in a rat. Biken J. 26, 155–160.

Yang, S. Y., Jensen, R., Folke, L., Good, R. A. and Day, N. K. (1974). Complement deficiency in hamsters. Fed. Proc. 33, 795.

Zabeau, M. and Voss, P. (1992). Selective restriction fragment amplification: a general method for DNA fingerprinting. European Patent Application EP 0534858.

---

List of Suppliers

**Allentown Caging Equipment Co., Inc.**
Route 526
Allentown, NJ 08501-0698
USA
Tel.: +1 609 259 7951
Fax: +1 609 259 0449
Equipment; IVCs.

**Charles River Laboratories**
251 Ballardvale Street
Wilmington, MA 01887-1000
USA
Tel.: +1 978 658 6000
Fax: +1 978 658 7132
www: http://www.criver.com
Equipment; diagnostic services; murine serological reagents.

**Anmed/Biosafe, Inc.**
7642 Standish Pl.
Rockville, MD 20855
USA
Tel.: +1 301 762 0366
Fax: +1 301 762 7438
Health monitoring.

**Ehret**
Fabrikstrasse 2
79312 Emmendingen
Germany
Tel.: +49 7641 92650
Fax: +49 7641 47972
Equipment; ventilated cabinets; isolators.

**Becker & Co.**
Hermanstrasse 2-8
D-44557 Castrop-Rauxel
Germany
Tel.: +49 2305 97304 0
Fax: +49 2305 97304 44
Ventilated cabinets; gnotocages.

**Labor für biomedizinische Diagnostik**
Feodor-Lynin-Str. 26
D-30625 Hannover
Germany
Tel.: +49 511 548885
Fax: +49 511 548886
Health monitoring.
Lab Products
742 Sussex Avenue
Seaford DE 19973
USA
Tel.: +1 302 628 4300
Fax: +1 302 628 4309
www.http://www.labproductsinc.com
Equipment; IVC system.

Metall and Plastik
Bodmanerstrasse 2,
D-78315 Radolfzell
Germany
Tel.: +49 7738 9280 0
Fax: +49 7738 9280 0
Equipment; isolators.

Microbiological Associates Inc.
9900 Blackwell Road
Rockville, MD 20850
USA
Tel.: +1 301 738 1000
Fax: +1 301 738 1605
Health monitoring.

Microflow-MDH Ltd.
Walworth Road
Andover
Hampshire SP10 5AA, UK
Tel.: +44 1264 36211
Fax: +44 1264 356452
Ventilated cabinets; isolators; hydrogen superoxide generator.

Perimmune, Inc.
1330 Piccard Drive
Rockville, Maryland 20850
USA
Tel.: +1 301 921 0422
Fax: +1 301 977 1403
www.www.perimmune.com
Murine serological reagents.

RCC Biotechnology and Animal Breeding
Wölferstr. 4
CH-4414 Füllinsdorf
Switzerland
Tel.: +41 44 61 906 4242
Fax: +41 44 61 901 2565
Transgenic and cryopreservation service; health monitoring.

REC BioZone
PO Box 246
Margate, Kent CT19 9AG
UK
Tel.: +44 1843 232090
Fax: +44 1843 228875
Equipment; IVCs; biocontainment.

Research Animal Diagnostic and Investigative Laboratory (RADIL)
University of Missouri
1600 East Rollins Street
Columbia
Missouri 65211
USA
Tel.: +1 532 882 1419
Fax: +1 532 884 4803
Health monitoring.

Scanbur A/S
Gl. Lellingegård
Bakkeledet 9, Lellinge
4600 Køge
Denmark
Tel.: +45 5682 0221
Fax: +45 5682 1405
Equipment; ventilated cabinets; isolators.

Tecniplast
Gazzada 21020
Buguggiate (VA)
Italy
Tel.: +39 332 459264
Fax: +39 332 459278
Equipment; IVCs.
Thoren Caging Systems, Inc.
P.O. Box 586
Hazleton
PA 18201, USA
Tel.: +1 717 455 5041
Fax: +1 717 454 3500
Equipment; IVCs.

UNO Roestvaststaal BV
Postfach 15
6900 Zevenaar
Niederlande
Tel.: +31 316 524451
Fax: +31 316 523785
Equipment; IVCs; Biocontainers.