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1 Management of Immunocompromised and Infected Animals

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◆◆◆◆◆◆ INTRODUCTION

Studies of the immune system have started with the vaccination of outbred animals, the examination of the immune response with respect to specific antibodies, immune cells and resistance to the corresponding infections. Inbred and congenic lines were developed, giving insight into the major histocompatibility complex (MHC) and allowing more detailed questions about the function of the immune system. In the course of inbreeding especially, many natural variants were found, including immunocompromised animals that served as models for human and animal disorders of the immune system. In the last decade a vast number of immunocompromised strains was created by genetic manipulation (Kühn and Schwenk, 1997; Croy *et al.*, 2001; Yu and Bradley, 2001) either directly or via the loxP-cre system allowing organ-specific deletions by the use of the respective promoters (Ray *et al.*, 2000; van Duyne, 2001). In addition, systems for artificially inducible deletions have been developed (Jaisser, 2000).

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In many areas of immunity, natural and induced immunodeficient variants have been used to study very different aspects of immunity such as T-cell function (Wang *et al.*, 1997; Croy *et al.*, 2000), autoimmunity (Shevach, 2000), cytokine effects (Kopf *et al.*, 1995; Assemann *et al.*, 1999; Kawachi *et al.*, 2000), cancer development (Bankert *et al.*, 2001; Berking and Herlyn, 2001; Recio and Everitt, 2001) and pathogenesis of infections (Mosier, 1996; Hess *et al.*, 2000; Schaible and Kaufmann, 2000). From these studies, the high complexity and considerable redundancy of the immune system became evident. A large number of the transgenic mutants used particularly in the field of immunology are immunodeficient, being more or less susceptible to infections. Particularly in these animals, known pathogens as well as ubiquitous micro-organisms may drastically reduce the breeding efficiency, endanger the maintenance and may counteract experimental manipulation. Reconstitution with immunocompetent cells and the use of antibiotics may only be a preliminary help. Finally, optimal hygienic standards are indispensable for these animals. The prerequisites are corresponding housing facilities, well-trained personnel, experience in re-derivation and the continuous monitoring of the stocks.

In addition to the microbiological status, the genetic background of the immunodeficient variant may strongly affect experimental results. Therefore, variants produced naturally or artificially have to be introduced into the desired background by a series of time-consuming backcrosses. Marker-assisted selection, also termed 'speed congenics', can accelerate this procedure.

Environmental conditions, food, water, handling, light cycle, etc. can alter experimental results. These factors have to be considered and standardized as far as possible. Furthermore, in some immunodeficient animal strains an ulcerative colitis (inflammatory bowel disease) may develop spontaneously (Bregenholt and Claesson, 1998).

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. 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 were established, such as caesarean derivation and subsequent raising in isolation. 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 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 animals (or humans) or the results of animal experiments.

Influence of micro-organisms on research results

It is a well-known fact 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, is frequently underestimated, but it may also be devastating because infections often remain undetected. Scientists in general are not well informed of such influences on their research. Only a small percentage of detected complications have been published. The literature is scattered across diverse scientific journals, and many articles are difficult to locate. To address the problem, conferences have been held on viral complications on research, and the knowledge available has been summarized in conference proceedings (Bhatt *et al.* 1986a; Hamm, 1986). The literature available has later repeatedly been reviewed (Lussier, 1988; National Research Council, 1991; Hansen, 1994; Baker, 1998; Nicklas *et al.*, 1999).

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 activate latent infections resulting in, for example, 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 that 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 MHV). This influences the disease rate and the mortality, as well as the type and severity of pathological changes. As another example, the immunosuppressive variant of 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 parameters, 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, or B cells, or specific sub-populations are influenced. Therefore, most virus infections and infections with bacteria or parasites are detrimental for immunological research and must be avoided.

Some micro-organisms have a specific effect on enzymatic or haematological parameters. LDV can induce up to 100-fold increase in the activity of 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 and immunodeficient animals are usually more sensitive to infections than 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 infectious studies are free from adventitious infections. An adventitious organism due to immunomodulation may influence the infection in question and, therefore, result in increased or reduced resistance to experimental infection. Micro-organisms resulting from a natural infection might contaminate viruses, bacteria, or parasites that are passaged in laboratory animals. Spontaneous infections may lead to false conclusions. For example, the first isolations of Sendai virus were made from mice that had been inoculated with diagnostic materials from humans and swine. In subsequent years, evidence accumulated to show that an indigenous virus of mice had been isolated (National Research Council, 1991).

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 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 routine 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. It may therefore 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) should be monitored to prevent the introduction of agents into a facility.

Different methodologies are applicable to detect infections in a population. In general, absence of clinical signs has only limited diagnostic value because most infections in rodents are subclinical. While most parasites (ectoparasites, helminths, protozoans) are usually detected by microscopic methods, culture methods are still preferred for detecting most bacteria or fungi. Serology is the most commonly used routine method indirectly to detect viral and also bacterial infections (e.g. *Mycoplasma pulmonis*) in a population by demonstration of antibodies. Serological methods bear the risk of false-positive or false-negative reactions, and unexpected serological results should therefore be confirmed by a second (confirmatory) method, by a second laboratory, or by monitoring additional animals. Molecular methods have become increasingly important during the last few years (Compton and Riley, 2001). They are preferred for the detection of agents that are fastidious or cannot be cultured easily (e.g. *Pneumocytis carinii*, *Helicobacter* sp., viruses causing persisting infections or during the acute phase of an infection). Molecular methods are usually more sensitive than traditional methods. Meanwhile, a very broad spectrum of molecular tests is available for every pathogenic organism of interest (with the exception of prion diseases).

There is always a risk that infectious agents might be introduced into animal facilities, 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 persons need access to the animals). Simulation experiments have shown that small and frequent samples are more suitable to detect an infection than larger samples taken at less frequent intervals (Kunstyr, 1992).

Although efforts have been made since the 1960s, a universal testing strategy or reporting terminology for clear and consistent definition of pathogen status in rodent population does not exist. The need for health surveillance programmes is generally accepted, but there is a great diversity of opinions about their design. Each institution selects its own list of pathogens, test procedures, animal sampling strategy, frequency of sampling, and reporting terminology, and the terms used vary greatly in precision and meaning (National Research Council 1991; Lindsey, 1998; Jacoby and Homberger, 1999). Usually, an individual programme is 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 physical conditions and layout of the animal house, husbandry methods and sources of animals. Number and quality of personnel as well as finances further influence the programme. 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). Some aspects to consider when establishing a health surveillance programme have repeatedly been provided (Nicklas, 1996; Weisbroth *et al.*, 1998). Detailed recommendations for monitoring of breeding or experimental colonies of rodents and rabbits were published by the Federation of European Laboratory Animal Science Associations (FELASA) (Nicklas *et al.* 2002).

The term most frequently used to describe the microbiological quality is 'specified pathogen free' (SPF), but this term requires explicit definition every time it is used. It means that the absence of individually listed micro-organisms has been demonstrated for a population by regular monitoring of a sufficient number of animals at appropriate ages by appropriate and accepted methods. SPF animals originate from germ-free animals. These are usually associated with a defined microflora and subsequently lose their gnotobiotic status by contact with environmental and human micro-organisms. Such animals are bred and housed under conditions that prevent the introduction of unwanted micro-organisms. SPF animals are morphologically and physiologically 'normal', and well suited for modelling the situation of a human population.

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 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. In general, the number of animals to be monitored is determined by the expected prevalence of an agent in a population. 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. This is (theoretically) sufficient to detect an infection with a 95% probability if at least 30% of a population is infected. Monitoring animals of different age is useful, because younger animals often have a greater parasitic or bacterial burden, whereas older animals (≥3 months) are more suitable to detect viral infections. Clinically ill animals are an import source of information and should be submitted to monitoring in addition to scheduled samples. Selwyn and Shek (1994) and Clifford (2001) have discussed strategies for sampling and calculation of sample sizes.

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. These may not be able to produce sufficient amounts of antibodies so that their status can best be evaluated 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 agents to be monitored. For example when using sentinels to monitor immunodeficient animals, the sentinels must be initially free from *Pneumocystis carinii* or *Staphylococcus aureus*. The sentinel animals must be housed for a sufficiently long time in the population that is to be monitored to develop detectable antibody titres (for serology) or parasitic stages. It is common to house sentinels in a population at least for 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 generally 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 can be more sensitive to an agent and thus more likely to develop overt 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 mouse hepatitis virus (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 may cause high mortality with typical skin lesions in C3H, 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 or other immunosuppressive drugs (e.g. cyclophosphamide) to suppress the immune system was recommended. This results in overgrowth and easier

direct detectability of bacterial pathogens. However, such tests have lost importance as direct demonstration of micro-organisms can now be performed more easily by means of molecular methods such as 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 breeders) are to be used as sentinels in order to obtain reliable results. With respect to barrier protected facilities it is advisable 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 the sentinel cages.

During the last decade additional housing systems such as filter-top (microisolator) cages, individually ventilated cages or filter cabinets emerged. They offer the advantage of separating small populations from each other and are frequently used for housing immunodeficient, immunosuppressed, or infected animals. If handled properly, they prevent transmission of infectious agents very efficiently. Each isolator or microisolator cage must therefore be considered a self-contained microbiological entity. Health monitoring under such housing conditions as well as monitoring of isolator-housed animals can be conducted properly only by the use of sentinels. Due to limited space, less than the recommended numbers 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 three to five animals per isolator will be available for monitoring.

If animals are housed in micro-isolators or in individually ventilated cages, sentinels must be housed in filter top cages like other animals. When cages are changed in changing cabinets, soiled bedding from several cages is transferred into a specific cage that is then 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, on the use of the population, and on the level of risk of infection for the population. Naturally, economic considerations do play a role as well. It is stated in the FELASA recommendation (Nicklas *et al.*, 2002) that monitoring be conducted at least quarterly. Most commercial breeders of laboratory rodents monitor more

frequently (every 4–6 weeks). In most multipurpose units housing of immunodeficient or infected animals, a more frequent monitoring is also 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. three to five 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 often (monthly), whereas testing for unusual organisms like K-virus or polyoma virus can be done less frequently (e.g. annually). Results obtained from monitoring sentinels are valid for all animals of the same species within a population, irrespective of the experiment or animal strain. Independent from animals that are scheduled for monitoring, all animals with clinical disease should be submitted for direct examination for micro-organisms (bacteria, parasites, viruses) and for histopathology.

Agents

A decision has to be made in each facility 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; Waggie *et al.*, 1994; Nicklas *et al.*, 2002) and can be used for guidance. Monitoring for all the agents mentioned (mycoplasma, bacteria, bartonellas, fungi, spirochaetes, protozoans, helminths, arthropods) on a routine base is neither realistic nor necessary. The most important micro-organisms are those that are indigenous and pose a threat to research or to the health of 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 the presently available methods. Other micro-organisms may be less important as they are unlikely to occur in good quality rodents due to repeated re-derivation procedures (e.g. *Brucella, Erysipelothrix, Leptospira, Yersinia*). Most cestodes are unlikely to be found, since they require an intermediate host. In the case of immunocompromised animals or in infectious experiments, however, monitoring for a comprehensive list of micro-organisms is reasonable. Various micro-organisms that usually do not cause clinical signs in immunocompetent animals (e.g. *Staphylococcus aureus, Pseudomonas aeruginosa, Pneumocystis carinii*) may cause serious problems in immunodeficient animals. Even agents such as *Burkholderia gladioli* may cause clinical signs in severely immunodeficient animals (Dagnaes-Hansen *et al.*, 1991). It is thus necessary to monitor immunodeficient animals not only for strong or weakly pathogenic organisms, but also for opportunistic pathogens or commensals. Micro-organisms with low pathogenic potential can cause clinical signs of diseases if animals are infected with several agents (e.g. KRV and *Pasteurella 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. This list is easiest to establish for viruses. 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 carried out selectively by serological methods. Only few exceptions exist, such as parvo-viruses, which cross-react in indirect immunofluorescence or ELISA tests (Jacoby *et al.*, 1996) and at present cannot always be identified unequivocally. Assays using recombinant antigens that have an increased specificity are under development. 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) (Ball-Goodrich and Johnson, 1994; McKisic *et al.*, 1995; Jacoby *et al.*, 1996; Ball-Goodrich *et al.*, 1998). One can expect 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 a hygiene problem and are therefore eradicated from rodent colonies. Some protozoans like trichomonads are occasionally detected 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 to wild rodents. The most complex problems exist for bacteria. In contrast to viruses their importance for laboratory animals is usually estimated on the basis of their ability to cause 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 *Pasteurella 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 (Nicklas *et al.*, 2002) 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 re-infection. The same holds true for several members of the Enterobacteriaceae (*E. coli, Klebsiella,*

Proteus), Staphylococcus aureus and *Pseudomonas aeruginosa* for which humans serve as a reservoir. Another problem arises from the fact that many bacteria are presently being reclassified, resulting in changes of their names. For example, the mouse specific organism known as '*Citrobacter freundii* 4280' has been reclassified as *Citrobacter rodentium* (Schauer *et al.*, 1995). Whole genera have been renamed, and additional bacterial species have been detected, such as *Helicobacter hepaticus, H. muridarum, H. bilis,* and *H. typhlonicus* (Lee *et al.*, 1992; Fox *et al.*, 1994, 1995; Franklin *et al.*, 1999). 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 times; 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 and commercial breeders 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 to detect bacterial pathogens in an animal. Bacterial cultures should be made for the respiratory tract (nasal cavity, trachea, lungs), the intestinal tract (small and large intestine) and urogenital tract (vagina respectively 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 living animals must be shipped to the monitoring laboratory. Therefore, many laboratories monitor only serologically. Although serological methods exist to detect some bacterial infections, these are not generally accepted and only 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

each monitoring programme. During recent years PCR has been increasingly used for the detection of slowly growing or fastidious bacteria such as *Helicobacter* (Riley *et al.*, 1996; Mähler *et al.*, 1998) or fungi such as *Pneumocystis carinii* (Rabodonirina *et al.*, 1997; Weisbroth *et al.*, 1999). Meanwhile methods are available for almost all agents (Feldman, 2001).

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.

The design of modern laboratory animal buildings is mainly based on microbiological concepts aimed at the prevention of infections. These measures are responsible for a high percentage of expenses 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 transmission of micro-organisms within a colony. This can be influenced by the management and the housing system.

Animals

The greatest risk of contamination to any animal arises from another animal of the same species. Most facilities are multi-purpose and must therefore house a variety of strains coming from different breeding units. In addition, many specific strains or genetically modified animals are available only from research institutes. Animals are therefore the most important risk factor, even though their quality has constantly been improved during the last few decades. The importance of animals as

sources of infections becomes obvious from a survey conducted in the USA in 1996. In this survey of 72 of the top 100 institutional recipients of NIH funds, MHV was 'on campus' in nearly 60% of the reporting institutions. Pinworms were reported to be present in >30% and parvo viruses in >25% of 'SPF' colonies (Jacoby and Lindsey, 1997).

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 genetically modified 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 a few sources of well-known microbiological status, and if these animals have been protected from contamination during shipment. Direct transfer of such animals without quarantine into an experimental unit can 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 caesarean section. Outbred mice are commonly 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 virus may be eliminated completely. Immunodeficient animals may, however, shed infectious virus for longer periods of time, or may be infected persistently (Barthold *et al.*, 1985; Weir *et al.*, 1990; Compton *et al.*, 1993; Gaertner *et al.*, 1995; Rehg *et al.*, 2001). Animals known to be infected must always be housed in isolation. This can best be done in flexible film isolators or, if proper handling is guaranteed, in microisolator cages or in individually ventilated cage racks.

The principles that are important for designing a quarantine programme have been thoroughly discussed by Rehg and Toth (1998).

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 a high percentage of these thereby become contaminated. Such materials can be stored frozen without loss of infectivity and may be hazardous for humans or for laboratory animals even after decades. Immunodeficient animals (e.g. nude mice or rats, $Prkdc^{scid}$ mice) are often used in xenotransplantation studies and are at risk to infections transmitted via transplanted tissue.

The problem of viral contamination in biological materials became obvious from studies 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 after animal passages was found by Nicklas *et al.* (1993a). 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 be transmitted by other contaminated tissues or body fluids such as monoclonal antibodies (Nicklas *et al.*, 1988) or viruses (Smith *et al.*, 1983). Two recent outbreaks of ectromelia in the USA both resulted from use of contaminated serum samples (Dick *et al.*, 1996; Lipman *et al.*, 2000). In colonies of genetically modified animals, ES-cells, sperm and embryos should be considered as potential sources of infection. ES cells in particular are at increased risk of infection because they require growth factors that are usually supplemented by co-culture with primary mouse cells (Hogan *et al.*, 1994). Contamination of biological materials is not restricted to viruses. *Mycoplasma pulmonis* and other bacterial pathogens like *Pasteurella pneumotropica* or *Coxiella burnetti* (Criley *et al.*, 2001) have been found as contaminants. Additional pathogens (*Eperythrozoon* sp., *Haemobartonella* sp., *Encephalitozoon* sp.) can contaminate biological materials after animal-to-animal passages (Petri, 1966; National Research Council, 1991) and thus may be transmitted to recipient animals.

Biological materials have traditionally been tested for contaminating agents using the mouse or rat antibody test (MAP or RAP test). Meanwhile, PCR tests have been established to replace the MAP test as the preferred test for detecting viral contaminants in biological materials (Compton and Riley, 2001).

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 which is present in humans who have access to a barrier unit might colonize the animals sooner or later. 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 in long-term experiments, that are known to have an increased sensitivity to infection with bacteria of human origin (*Staphylococcus aureus, Klebsiella pneumoniae, Escherichia coli*, etc.)

should preferably be housed in isolators or microisolators (respectively 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 filters or by insect-electrocuting devices. Crawling insects such as cockroaches are more difficult to control, and cannot definitely be excluded. 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, which 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 rodents

Since serological testing was introduced in the 1970s, many laboratories have evaluated the viral status of murine colonies. Managing directors of animal facilities had to learn techniques for the prevention, control and eradication of infection, and the 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 almost entirely been 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 quality animals for research. However, this progress of eradication has not happened 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 (1991) indicates that the majority of colonies at that time was infected with three or four viruses. However, most facilities still house at least small numbers of animals that are infected or have an unknown status. Many small or decentralized facilities do not even monitor at all. In a retrospective study among French facilities the prevalence rate of some agents decreased, but some were still found to harbour viruses during the last years of the study (Zenner and Regnault,

2000). In a survey conducted in the USA among major biomedical research institutions MHV and mites were reported to be present in more than 10% of SPF mouse colonies, and more than 25% were reported to be positive for pinworms and parvoviruses. As expected, the prevalence of infections was even higher among non-SPF mice (Table 1). More than one-half of the replying institutions had endoparasites and MHV 'on the campus'. More than 10% were positive for at least seven other viral and bacterial infections. Surprisingly, serological evidence of ectromelia virus and lymphocytic meningitis virus (LCMV) were also reported (Jacoby and Lindsey, 1997, 1998). A similar profile to that of mice was revealed for rats (Table 2).

Table 1. Prevalence of infections in colonies of laboratory mice (Jacoby and Lindsey 1997, 1998)

Agent	Percentage of positive 'SPF' colonies	Percentage of positive 'non-SPF' colonies	Agent 'on campus' % colonies positive
Mycoplasma	3	17	12
Helicobacter	13	9	18
Mites	17	38	36
Pinworms	33	68	63
Adenovirus	2	8	8
Sendai	0	21	15
PVM	3	22	18
Reo	3	19	15
TMEV	4	35	29
Rota	6	28	24
MHV	12	74	57
Parvo	27	40	46

Table 2. Prevalence of infections in colonies of laboratory rats (Jacoby and Lindsey 1997, 1998)

Agent	Percentage of positive 'SPF' colonies	Percentage of positive 'non-SPF' colonies	Agent 'on campus' % colonies positive
CAR bacillus	6	22	19
Mycoplasma	0	36	26
Mites	4	12	12
Pinworms	33	68	53
Sendai	2	23	18
PVM	9	28	22
Reo	3	8	8
TMEV	8	18	15
Rota	2	7	4
Corona	9	38	32
Parvo	27	33	33

Various agents are still prevalent at a low level. They can emerge unexpectedly as seen a few years ago when a sudden outbreak of ectromelia was observed in the USA (Dick *et al.*, 1996). The situation is very similar for bacterial pathogens and parasites. Most of them were eradicated when principles of gnotobiology had been introduced into laboratory animal science. A few parasites (pinworms, mites, protozoans) are still endemic in various rodent colonies. Today, 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 *Mycoplasma pulmonis* are detected more often. Most experimental and some commercial breeders' colonies are positive for Pasteurellaceae like *Pasteurella pneumotropica* and *Actinobacillus muris*. The real prevalence of organisms belonging to this family is not definitely known due to difficulties in identification. The situation is also unclear for *Helicobacter* species because many colonies are not sufficiently monitored. It is to be expected that these micro-organisms are also widespread in laboratory rodents. It is, therefore, extremely important that germ-free, or gnotobiotic animals, rather than SPF animals, are used for hygienic rederivation to avoid this problem in future.

A number of additional disease agents, for example group B and G streptococci, *Staphylococcus aureus, Haemophilus parainfluenzae, Corynebacterium* 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, which are more likely to be transmitted by humans. These infections have been named 'post-indigenous diseases' (Weisbroth, 1996). While agents such as '*Corynebacterium bovis*' might be tolerable in immunocompetent animals, clinical disease caused by these bacteria may be seen in immunodeficient animals (Clifford *et al.*, 1995; Scanziani *et al.*, 1997, 1998).

The presence of infectious agents, even if they are of low pathogenicity, may become a problem if animals from different sources are co-maintained. 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

Natural variants (mutations, infectious agents)

Naturally occurring immunodeficient mouse strains express a variety of genetic defects in myeloid and/or lymphoid cell development. These strains have served as and are still valuable models for studying immune cell differentiation, mechanisms of transplant rejection, etc. Some of the most commonly used mutants are nude ($Foxn1^{nu}$), severe

combined immunodeficiency ($Prkdc^{scid}$), beige ($Lyst^{bg}$) and X-linked immunodeficiency (Btk^{xid}). Sufficient information on the different variants produced by nature can be obtained from an ILAR Committee report (1989), Hedrich and Reetz (1990), Lyon *et al.* (1996), or more specifically by searching for defined mutations in databases, such as Mouse Genome Database (*http://www.informatics.jax.org*) and RATMAP (*http://ratmap.gen.gu.se*). Aside from their immunodeficient status, i.e. their inability to eliminate or neutralize foreign substances, some of the 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, while C57BL/6 and related strains succumb to an infection with *Streptobacillus moniliformis*, AKR, BALB/c, DBA/2, and other mice survive, while BALB/c never show any sign of disturbance, nor even produce antibodies against this organism (Wullenweber *et al.*, 1990).

Induced immunodeficiencies

Aside from these immunodeficiencies there are other means to modulate the immune status of mice and rats. These induced deficiencies such as thymectomy, lethal or sublethal irradiation, depletion of various subpopulations of immune cells by antibodies require exactly the same management procedures as for any immunodeficient animal.

Genetic manipulation

The advent of transgenic rodent technology by transferring and overexpressing foreign genes under the control of specific vectors as well as directed mutagenesis by silencing specific genes has opened up new avenues for the study of innumerable factors affecting 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://tbase.jax.org/*; mouse knock out and mutation database, *http://research.bmn.com/mkmd*). Again, as indicated above, identical phenotypes must not necessarily indicate identity of the genes. While in many respects phenotypically alike, the genetic factors controlling the expression of $Prkdc^{scid}$ and $Rag1^{tm}$, respectively $Rag2^{tm}$ deficient mice have been shown to be different. On the other hand, silencing of exon 3 of the *Whn*-gene has produced exactly the same phenotype as in $Foxn1^{nu}$-mice providing evidence that the fork head transcription factor is responsible for the nude and athymic phenotype (Nehls *et al.*, 1996).

PCR protocols by which mice carrying an induced mutation can be distinguished from normal wild type mice have been published in the respective descriptions. Some are available on the World Wide Web, e.g.

those maintained at the Jackson Laboratory (*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 laboratories, which comply with the national requirements of the respective host country for the use of genetically modified animals.

◆◆◆◆◆◆ MANAGEMENT OF COLONIES

Housing systems

The initial descriptions of housing systems for small rodents have not lost their principal validity (Spiegel, 1976; Otis and Foster, 1983; ILAR Committee, 1989; Heine, 1998) although many refinements have since then been introduced. On the basis of scientific demands, international standards and the risk for the own animal facility, the decision for the adequate hygienic status has to be made. In principle, this could be:

- *Germ-free*, designating a status in which no micro-organisms are present except those integrated into the genome.
- *Gnotobiotic*, in which the animals have a well-defined and specified flora, consisting mainly of anaerobic bacteria supporting the metabolism and possibly fertility. In addition, the gnotobiotic flora may induce some resistance to ubiquitous micro-organisms:
  SPF: specified pathogen-free, which describes by definition animals being free of pathogens to be specified.
- *Quarantine*, which was originally used for overcoming the potential latency period of infections, in this context, however, includes a different but not an acceptable SPF status, in particular for newly introduced strains from other institutions.
- *Infectious*, denominates the status of animals either infected naturally or artificially which could transmit pathogens not found in the animal's own colony or being a risk for humans or other species.

Isolators

Static micro-isolators are partially perforated boxes with a tight filter medium, covering the perforation and a cover, which has to be sealed (Kraft, 1958). They provide a micro-environment which is protected from adventitious contamination from outside. Micro-isolators are still in use (e.g. Han-Gnotocage[1]) for the transport of germ-free, gnotobiotic and SPF-founder animals. These can also be used for the short-term housing of germ-free fosters in a laminar flow cabinet. The disadvantage of these micro-isolators, however, is the impeded intracage ventilation. Due to an increase in humidity, ammonia and carbon dioxide concentrations and

1 HAN-Gnotocage, Firma EBECO, Hermannstrasse 2-8, D-44579 Castrop-Rauxel, Germany.

with increasing animal density this intracage ventilation becomes intolerable.

Ventilated isolators in the *positive pressure* version are indispensable for breeding and maintaining germ-free and gnotobiotic animals. They consist of a closed construction with a HEPA-filter unit for air supply, a valve or a filter in the exhaust, long-arm gloves and a chemically sterilizable lock for interconnection to the supply chamber (Trexler, 1983). For chemical sterilization of the isolator and the lock, freshly diluted peracetic acid[2], alkaline buffered peracetic acid[2] or hydrogen peroxide gazing should be used. In the case of pinworm contamination, an additional treatment cycle with an effective disinfectant (e.g. Chlorocresol[3]) should be carried out. Most of the required materials can be autoclaved in the supply chamber with control of the heating process by indicators (paper[4] and maxima thermometer) and in the retrograde by bioindicators (*Bac. stearothermophilus, Bac. subtilis* spore strips[5]). If dietary problems arise as a consequence of the food sterilization, gamma-irradiated diet (50 kGγ) can be used after chemical sterilization of the outside of the vacuum bags.

Germ-free animals are used for special experiments, for example to test the influence of the gut flora (Hirayama, 1999), the effect of lipopolysaccharides on the immune system (Enss *et al.*, 1997) and as a back-up for foster mothers, which may be necessary to renew the gnotobiotic status. In this context, it has to be stressed that in germ-free mouse and rat strains very often a mega-caecum is found (Wostmann *et al.*, 1973), productivity can be decreased or lost and, when exposed to the outside environment, these animals may fall ill and die.

Gnotobiotic animals are normally derived from germ-free animals by oral application of a gnotobiotic flora[6] consisting mainly of anaerobic, well-defined micro-organisms (Dewhirst *et al.*, 1999). The reproductivity in this hygienic status may be restored and the resistance to outside environment may be improved in comparison to germ-free animals (Heidt *et al.*, 1990; van den Broek *et al.*, 1992). Therefore, this status can be recommended for immunocompromised strains – at least for their breeding stocks – and for foster mothers used for embryo transfer. A gnotobiotic status can only be preserved in isolators, a fact that may restrict the expansion of colonies. This limitation may be circumvented by the use of individually ventilated cage systems, although contamination with other micro-organisms cannot be excluded totally.

The equipment of most commercially available isolators allows the alternative use in positive or negative pressure. Isolators in the *negative pressure version* protect in the first line the environment from infections inside the isolator by an HEPA-filter in the exhaust. In combination with

2 Peracetic acid: Kesla Pharma Wolfen GmbH, Thiuramstrasse 2, D-06803 Greppin, Germany.
3 Chlorocresol (Neopredisan ®): Menno-Chemie Vertrieb GmbH, D-22850 Norderstedt, Germany.
4 Indicator-paper: BAG Biologische Analysensysteme GmbH, D-35419 Lich, Germany.
5 Bio indicators: Werner, MBS, Untere Jasminstaffel 3, D-88069 Tettnang, Germany, Apex Laboratories, P.O. Box 794, NC 27502-0794, USA.
6 Gnotobiotic flora: Taconic: 273 Hover Avenue, Germantown, NY 12526, USA.

the use of a waste chamber, their use is obligatory for infectious experiments with high-risk pathogens and may be recommended for stocks of risk in quarantine if no appropriate barrier system is available.

Individually ventilated cages (IVCs)

By direct ventilation of individual cages with HEPA-filtered air, the presumptions for long-term bio-containment on the cage level can be accomplished. In addition, ventilation with *positive* pressure in the cages counteracts the leakiness of the system. *Negative* cage-pressure may prevent the escape of micro-organisms and allergens from the IVCs. The different aspects of ventilated cage systems are described in an overview by Lipman, 1999 and special topics are presented by Clough *et al.*, 1994, Perkins and Lipman, 1996, Hasegawa *et al.*, 1997, Tu *et al.*, 1997, Chaguri *et al.*, 2001, Gordon *et al.*, 2001, Höglund *et al.*, 2001, Reeb-Whitaker *et al.*, 2001 and Renström *et al.*, 2001.

Different versions are commercially available, in which the air is blown either directly into the cage or is passed through a wide mesh filter. In the latter, the intracage air velocity is lower but the desiccation of the bedding is reduced. In many systems the exhaust air passes a filter in the cage to retain dust from the exhaust pipes. In most of the different versions the intra-cage pressure can be adjusted to be *positive* or *negative*, respectively, allowing the use in different hygienic pretensions.

Handling of IVCs

This is the most critical and most underestimated procedure of running IVCs. Three different hygienic levels have to be considered:

1. The sterility level of the autoclaved material: *cage* with bedding, cover and lid as a whole, *diet* (or gamma-irradiated and outside sterilized) and *water* bottles, sterile transferred into the laminar flow changing station (in case of inside bottles).
2. The outside environment of the cage, e.g. the animal room.
3. The inside space of the cage containing the animals.

In a correct manipulation, these three levels have to be strictly discriminated. Several regimens for one (see Box) or two persons may be used.

The procedure of sterile handling of IVCs is labour intensive, however, it can be compensated at least in part by extending the cage change interval, due to the higher intracage ventilation. In addition, increasing the change interval reduces stress for the animals (Duke *et al.*, 2001; Reeb-Whitaker *et al.*, 2001).

IVCs can be used to breed and maintain animals within an SPF-unit to reduce the risk of contamination to the cage level at least theoretically and to improve the environmental conditions for the animals, which is of special interest in immunocompromised rodents. In addition, the personnel are fairly well protected from allergens and from smell if the exhaust of the IVCs is connected to the air outlet of the room. Problems may arise,

Handling of IVCs

1. Laminar flow bench is running (30 min in advance).
2. Fast-acting sterilization compound (Recommendation: Clidox[1]) is freshly diluted for gloves and bench table and (in a separate vessel) for the forceps that can be used.
3. A filter top cage, fully equipped and autoclaved, is placed on the bench.
4. Sterile diet (kept in a filter-top cage) is filled in using a sterile ladle with a long handle.
5. Sterile water bottle is inserted using sterile pincers – in case of inside bottle location – or after disinfection of nipple and grummet – in case of outside bottle systems.
6. The cage to be changed is placed into the bench.
7. After removing the hoods of both cages, animals are transferred into the new cage, using sterile forceps.
8. Lids and filter tops are replaced and both cages removed.
9. The work-place and the gloves are disinfected after each working phase with Clidox.
10. In case of infectious animals, a biohazard, class II laminar flow bench has to be used and contaminated cages have to autoclaved with filter tops in place.

however, in health monitoring which has to be aligned to the cage level. IVCs are also particularly useful when the prerequisites for an *SPF-unit*, except an autoclave, are lacking and in *experimental areas*, where easy access to the animals by the scientists is indispensable. In addition, IVCs can be of help to preserve the hygienic status of the individual colonies. Barrier closed *quarantine* IVCs in *positive* pressure are ideal for containment of animals from different sources in their respective environment. For quarantine without additional barrier system and *infectious experiments* IVCs are run with *negative* pressure. In addition, *sealed* IVCs were developed, however, it should be kept in mind that sealing may not be absolutely tight and therefore hazardous experiments should be performed in isolators.

SPF-unit

By definition, animals are free of specified pathogens. However, no declaration on residual micro-organisms is given, implying the probability of extensive differences from one SPF-unit to another (Heine, 1980; O'Rourke *et al.*, 1988; Boot *et al.*, 1996). Therefore, it must be considered that when transfering animals from one SPF-unit into another, additional micro-organisms can be introduced which may disturb the microbiological equilibrium, particularly in immunocompromised animals (Ohsugi *et al.*, 1996).

1 Clidox: Outside Europe: Pharmacel Inc., Naugatuck, Connecticut 06770, USA
Within Europe: Fi. Tecniplast, Gazzada 21020, Buguggiate (VA), Italy
Dilution: 1 part basic component, 5 parts water and 1 part activator
Ready for use after 15 minutes.

An SPF-unit is protected by a strict hygienic barrier system of air supply, materials, food, bedding and personnel (Otis and Foster, 1983; ILAR Committee, 1989; Heine, 1998). A conventional open caging system or IVCs may be used within the SPF-unit. After disinfection with formaldehyde or hydrogen peroxide (Krause *et al.*, 2001), gnotobiotic or SPF animals can be introduced via a chemical lock by external disinfection after covering the filter of the micro-isolator cage with a foil. *Standardized diet* is introduced by autoclaving whereby the diet has to be *fortified*, i.e. heat-sensitive vitamins are added in excess ensuring that sufficient amounts remain after heat treatment. The hardness after autoclaving must be controlled regularly. As an alternative, gamma-irradiated food (25 KGy) in vacuum bags can be introduced into the SPF-unit after external disinfection. The *drinking water* should be sterilized by heat, filtration or UV light and conserved by acidification (e.g. hydrochloric acid or acidic acid) to a pH of 3.0–2.5 or chlorination. For the latter, the pH should be adjusted to around 5 before adding stabilized hypochloride to reach 6–8 ppm of free chlorine (Leblanc, 2002). If problems arise from solubility of drugs to be added to the drinking water autoclaved tape water should be used. *Bedding* should be dust-free (<1% dust) and must be autoclaved with two or three vacuum cycles in advance. Pregnant females should be provided with nesting material such as autoclaved cellulose towels or nestlets (Van de Weerd *et al.*, 1997). The highest risk for the unit, however, is the *personnel* entering the barrier. Only a minimal number of well-trained caretakers (FELASA, 1995), having had no contact to external rodents for 4–7 days and being free of infections, should be allowed to enter the SPF-area.

The *microbiological status* of the SPF-area should be monitored regularly, sick animals removed from the unit and submitted to microbiological examination/necropsy and sentinels should be checked at fixed intervals (see Frequency of monitoring, p. 190). The regular disinfection of floors, walls and racks is strongly recommended.

Nowadays, the vast majority of small rodents is raised in SPF-units. If properly managed (see Box), such systems may stay 'clean' for many years. However, it should be heeded that the outbreak of an infection is unlikely to be restricted to single cages when conventional cages are used. With proper handling this may be prevented by the use of IVCs.

Quarantine

As a consequence of the genetic manipulation, the exchange of breeding stocks between institutions has rapidly increased. Because of the presumably different hygienic constitutions, the single stocks should be preserved in their own microbiological status until re-derivation can be performed. This can be achieved by the use of IVCs in *positive* pressure within a separate barrier-unit in *negative* pressure. *Quarantine* precautions should also be established in testing unknown cellular material provided to be introduced into animals for contaminations, which could be of risk to the animal facility (Yoshimura *et al.*, 1997 and Biological materials,

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. Animals caught outside the cage should be killed or isolated, if identification is possible.
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, the cage should not be redefined 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. 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.
9. 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.

p. 195). Animals, gamma-irradiated in an 'unclean' environment, should be submitted to quarantine as well.

Infections

Natural infections require a re-derivation in particular when rarely available stocks are concerned. IVCs in *negative* pressure should be used for containment until re-derivation is completed. In *experimental* infections the pathogenicity of the micro-organisms and the immune status of the animals determines the housing either in 'sealed' IVCs or in isolators.

Special considerations on immunocompromised animals

The consequences of gene manipulation on susceptibility to diseases cannot be predicted fully (Fernandez-Salguero *et al.*, 1995). Therefore, the aim when creating new lines should be the highest possible level of hygiene, especially of the foster mother, the manipulated embryos and the management of the new colonies. Of course, this is of special importance when revitalizing immunocompromised strains. In practice, special staff should be available for these tasks. The risk of contaminating the clean side via the embryos is low if proper 'washing' of the embryos is carried out (see later). Adherence to a strict regimen offers the possibility of raising transgenic animals at a level of hygiene adequate for immunocompromised animals, thus avoiding time-consuming re-derivation.

While immunocompetent animals are able to overcome most infections and to eliminate the pathogen, immunocompromised animals are often unable to cope with the pathogen and may be a source of infection for their entire life. Furthermore, in immunocompromised animals bacteria from the gastrointestinal tract can pass through the epithelial mucosa into the organism (Ohsugi *et al.*, 1996). The question arises as to whether the SPF standard is adequate for severely immunodeficient animals, or if a more stringent containment standard (germ-free, gnotobiotic), is advisable. It should, however, be taken into account that the immune system may depend on a general pre-stimulation which is lower in gnotobiotic and more so in germ-free animals. Therefore, experimental results should be interpreted with caution, when animals have been kept at different hygienic levels.

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 segregating 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 is sufficient information on the many mating systems for breeding rodents (Green, 1981; Silver, 1995).

Inbreeding

A unique advantage in working with mice and rats is the availability of standard inbred strains. By using this type of a strain, including an F1-hybrid, rather than an outbred stock or a strain with mixed genetic background, it is possible to eliminate genetic variability as a source of variation. By continuous brother by sister (BxS), or younger parent by off-

spring mating for a minimum of 20 generations this homogeneity can be obtained within a strain. After this period 98.02% of all loci within the genome of either animal of the particular strain should be homozygous. As of F12 the remaining heterozygosity within the (incipient) inbred strain will fall off by 19.1% per generation. The increase in homozygosity, respectively the loss of heterozygosity deviates from the expected value if there is any selective force (inadvertent or by purpose) towards a certain phenotype, or in case of mutations.

Congenic strains

In order to be able to identify 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. The simplest approach 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 is backcrossed 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. With the availability of the many DNA-markers nowadays available (*http://waldo.wi.mit.edu/rat/public/*; *http://www.informatics.jax.org/*; *http://ratmap.gen.gu.se/*; *http://www.otsuka.genome.ad.jp/ratmap/*) defining the locus of interest, or being tightly linked to it, other mating systems are in general no longer required. If a recessive allele in the homozygous state is lethal or induces sterility, a known heterozygote (as defined by genotyping) is back-crossed to the selected inbred (background) strain. Only when genotyping is difficult *in vivo* or in the aforementioned case cross–intercross matings have to be performed, whereby carriers are identified by the production of mutant offspring. Once identified, the heterozygote is crossed to the background strain and the resultant progeny again is intercrossed.

Speed congenics

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 about 1.5 years (Wakeland *et al.*, 1997). Apparently, with low density marker spacing of about 25 cM and screening only of male offspring of four litters at every generation a sufficient introgression is possible. This can be achieved after only five generations of back-crossing (Markel *et al.*, 1997; Wakeland *et al.*, 1997; Visscher, 1999). Moreover, the genome scan allows the identification of the chromosomal location of a transgene in *N*2 and may provide information on (unwanted) donor-derived regions. One has, however, to keep in mind that it is imperative that the marker set used for differentiation at the given interval does not exceed the upper limit (25 cM) and must be polymorphic unanonymously.

Propagation without inbreeding

Certain mutants cannot successfully be inbred or transferred to a specific inbred background in a fixed (homozygous) state. 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 supposed that these animals with a heterogeneous background are hardier, more productive, faster growing and have a longer life expectancy. For example, it is extremely difficult to maintain the athymic-nude mutation of the rat (Whn^{rnu}, $Whn^{rnu\text{-}N}$) on DA and LEW backgrounds. These colonies have to be propagated by constant back-crossing since nude offspring quite often do not surpass weaning (Hedrich, unpublished).

Many of the targeted mutants are, therefore, maintained on the variable, mixed background composed of the ES-cell donor and recipient strain genome and sometimes another 'prolific' strain or stock genome. If a mutation affecting the immune system cannot successfully be inbred due to effects on viability and fertility there is no other means but to maintain it on a segregating background or by back-crossing the mutation onto two different standard inbred strains and by producing homozygous mutant F1 offspring by mating mutant bearing heterozygotes 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 in background genotype, while F1 hybrids will match least.

Genotype preservation

Cryopreservation of embryos, gametes and even ovaries is an important tool to secure, archive and distribute strains or stocks of laboratory animals. The techniques for the different types of germplasm to be preserved vary greatly and often depend on the skills and equipment available in the various laboratories. While most publications refer to the mouse, reports on other species are scarce. This is mainly due to the exponentially increasing number of induced mouse mutations, either by gene targeting or by chemical mutagenesis, that have been and are under development.

Embryo freezing

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 not currently used. Despite certain improvements, the freezing of murine embryos is a time-consuming and cost-effective task. While outbred stock and hybrids in general respond to superovulation by gonadotrophins 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.

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 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 and freezing methods and freezing of other developmental stages have been reported (for an overview see Hedrich and Reetz, 1990).

Sperm freezing

Sperm freezing, although not well established, could assist in all cases where animal-holding space is limited. This primarily applies to, for example, ENU-mutagenesis programmes, or colonies of mice bearing mutations or transgenes. Although reports on sperm freezing associated with *in vitro* fertilization in mice claim that it is a successful means to alleviate the problems encountered with embryo freezing (Marschall and Hrabé de Angelis, 1999; Songsasen *et al.*, 1997; Sztein *et al.*, 2000), it is our experience that sperm freezing is reliable primarily in C3H mice, while results in other strains are rather poor (Sztein *et al.*, 2001).

Ovary freezing

The transplantation of ovaries is a technique to maintain mouse strains with breeding problems established long ago (Russell and Hurst, 1995). Splitting the ovaries into halves further eases the surgical transfer (Stevens, 1957). This modification also increases the probability of success by using up to four recipients. Recently, Stein *et al.* (1999) reported on the successful orthotopic transplantation of frozen-thawed ovaries into syngeneic ovariectomized recipients. Homozygous $Prkdc^{scid}$ mice will serve this purpose as well as syngeneic recipients (Hedrich, unpublished). This technique complements the techniques used in gamete banking.

Genetic monitoring

As well as differential fixation of alleles at early generations of inbreeding, mutations may alter the genetic constitution and thus the phenotype of an inbred strain. Many of the phenotypic differences detected between substrains have been shown to be due to these factors. Inadvertent outcrossing will alter a strain seriously, questioning its further use for research,

since results are no longer comparable and repeatable. It is thus of utmost importance to separate strains that are not immediately to be distinguishable by their phenotypic appearance. If, however, due to shortage in shelf space and separate animal rooms several strains must be co-maintained in one room, regular screenings for strain discriminating markers as well as the differentiating locus (in case of congenic strains) are indispensable.

Proper colony management is the first step towards the provision of authentic laboratory animals (see Box). As repeated handling of animals during regular caretaking cannot be avoided, there is always the risk of mistakes. An animal might inadvertently be placed into a wrong cage, or a false 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 structuring – nucleus colonies in a single (Festing, 1979) or parallel modified line system (Hedrich, 1990), pedigreed expansion colonies and multiplication colonies – should be self-evident, but 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 by 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 specific needs and may depend on the scientific purpose, the physical maintenance conditions and the laboratory equipment. 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 (as far as available), and which makes it possible 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, for example through Research Genetics Inc., Huntsville, AL, USA (*http://www.resgen.com*). Other sources for primers are also available through the World Wide Web (see earlier). 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 enables all strains maintained per separate housing unit to be identified. Unfortunately, this information is only partly available and not yet compiled in an accessible database. There are numerous publications and textbooks with protocols for PCR amplification and electrophoretic separation of the amplicons. Moreover, commercial suppliers of primers (e.g. Research Genetics) and of genetically modified animals (e.g. *http://informatics.jax.org*; check: Genes, markers and phenotypes, see

Polymorphism, or *http://www.jax.org/resources/documents/imr/protocols/index.html*) do provide PCR protocols. Nevertheless, it might be necessary to adjust temperature conditions as well as Mg^{2+} concentrations 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 polyacrylamide gel electrophoresis should be performed. As radioactive labelling with ^{32}P uses a kinase reaction and since the half-life of isotopes is relatively short, a silver staining procedure is recommended. Information on RFLP polymorphisms as determined by a Southern blot (Sambrook, 1989) using a specific probe may also be found in the mouse genome database (MGD) maintained by The Jackson Laboratory.

Nevertheless, the classical markers are still relevant and may need to be verified, and sometimes allow for a faster and less expensive phenotyping. In this context immunological markers are of prime importance. This group is composed of 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 the chapter by Czuprynski in Section II), flow cytometry (see the chapter by Scheffold *et al.* in Section I), immunodiffusion, ELISA (see the chapter by Yssel in Section III), immunohistochemistry ELISA (see the chapter by Ehlers *et al.* in Section II), using specific antibodies. The availability of antibodies depends on the specific marker and the species, with a broader spectrum available for mice. If it is too difficult to obtain or produce these antibodies certain markers might be demonstrated by applying published molecular biology techniques (see also: *http://www.informatics.jax.org/mgd.html*).

Further methods that can be applied easily and which depend on a specific phenotype may also be applied, as in the case of the lysosomal trafficking regulator (*Lyst^{bg}*, beige, expressing a pigmentation and platelet storage pool defect). The phenotype of homozygous beige mice can be determined by a prolonged bleeding time (20 min in homozygous *Lyst^{bg}* vs. 6 min in unaffected wild type or heterozygous controls), or a histochemical staining (checking for abnormal giant lysosomal granules detectable in all tissues with granule-containing cells; Novak *et al.*, 1985).

The determination of a profile is time-consuming and expensive, but strongly recommended as an initial check. In case of a variable segregating background genetic profiling is pointless as the typing results will only assist in determining the degree of heterogeneity. However, these results may provide hints on modifying genes, if the stock is being inbred and nearly homozygous.

Easy measures are still required to distinguish between those strains that are co-maintained and which clearly identify an outcrossing event. A critical subset of the markers (i.e. least amount of differentiating markers for a given strain panel) used to authenticate the strains maintained will provide reasonable information on the genetic quality of a strain. Unfortunately, with each strain added to a unit the number of markers in the critical subset increases. These critical subsets need to be verified at

regular intervals (every 3 to 6 months). The intervals and the number of animals to be tested are incremented to the number of strains co-maintained and to the size of each colony.

Irrespective of these methods one of the most powerful aspects of an inbred strain lies in the demonstration of its isohistogeneity. This is best demonstrated by skin grafting. The technique is easy to perform. It is, however, time-consuming because of an observation period of about 100 days (for a description of the techniques see Hedrich, 1990). In certain immunodeficient mutants (e.g. $Foxn1^{nu}$, $Prkdc^{scid}$, $Rag1^{tm}$, $Rag2^{tm}$) a direct demonstration of isohistogeneity is impossible, as these animals are incapable of mounting an allorecognition response. Transferring grafts from these immunodeficient animals to their syngeneic background strains can circumvent this.

◆◆◆◆◆◆ MANAGEMENT OF INFECTED COLONIES

Quarantine and natural infections

Animals with an unknown microbiological status have to be kept in isolation. The degree of isolation should be the same as that for infected animals as already described. The need for re-derivation of both categories is obvious.

Re-derivation

Hysterectomy

As shown for most infections, the vertical transmission of viruses, bacteria and parasites can be avoided by this procedure. The most difficult part of this procedure is to achieve timed pregnancy, especially in poor breeding strains. This method (see Box) is recommended if embryo transfer cannot be performed due to lack of equipment and trained personnel, or if a donor strain is refractory to superovulation. Hysterectomy has the additional risk of intrauterine vertical transmission of infections, which is to be considered higher in immunodeficient than in immunocompetent animals.

Embryo transfer

Embryo transfer was 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 mouse hepatitis virus (MHV) infection (Reetz *et al.*, 1988). The hygienic status of the foster mother should be of the highest level, especially when a new breeding unit is to be established. For routine procedures, the two-cell stage may be best suited because fertilization is no longer in question and a relatively high

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 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.

number of embryos can be collected. The animals are timed mated without or after previous superovulation (for details see Reetz *et al.*, 1988; Hogan *et al.*, 1994; Schenkel, 1995). The latter method normally induces the production of higher numbers of embryos (other than by normal mating) especially if prepuberal females are used, and allows synchronized matings. Embryos are flushed from the oviducts of plug-positive mice on day 1.5. They are selected for integrity (intact zona pellucida), washed at least four times at different locations and in sufficiently large volumes of media (approx. 2 ml), before transfer to a clean area where the transfer into the oviducts of pseudocyetic surrogate dams (day 0.5) is performed by a different person. Pseudocyesis can be induced by mating the surrogate dam with either a vasectomized, or a genetically sterile male (Silver, 1985). It should be mentioned that there are strain-specific differences with respect to the optimal amount of injected hormones and the number of embryos. Problems with superovulation are also known for most inbred rat strains.

The embryo transfer offers certain advantages versus hysterectomy. It avoids the risk of intrauterine vertical transmission of infections, and allows easier timing especially by superovulation and cryopreservation of surplus embryos.

Furthermore, new lines shipped as cryopreserved embryos can be transferred to surrogate dams of the present SPF status, thus avoiding time-consuming quarantine and rederivation procedures.

Preventive treatment

The preventive treatment of immunocompromised breeders with immunocompetent cells can be of help in the propagation of highly immunocompromised strains (Wang *et al.*, 1997; Kawachi *et al.*, 2000). To avoid graft-versus-host reactions, immunocompetent cells of F1 hybrids of the strain to be reconstituted with an immunocompetent strain should be used. For reconstitution of *nude* mice, thymus homogenates can be injected intraperitoneally to overcome their defect. For homozygous SCID, RAG or Gamma-c mice, the injection of F1 spleen cells i.p. ($1–2 \times 10^7$), or bone marrow cells ($2–5 \times 10^6$) i.v. into juvenile animals improves their constitution and thus marks them as suitable breeders (Mossmann, unpublished).

Therapeutic treatment

In general, the administration of therapeutics influences the outcome of animal experiments and cannot be considered as a means to replace the improvement of hygienic standards. However, therapeutic treatment may be unavoidable after gamma-irradiation and in immunocompromised strains if the latter have to be maintained in 'dirty' conditions until rederivation is completed (Macy *et al.*, 2000). The success of treatment depends on several criteria: a correct diagnosis including antibiotic resistance (Hansen and Velschow, 2000); the consideration on species-specific toxicity; adverse reactions of the therapeutic; and an optimal dosage and regimen of application and accompanying hygienic procedures. Unfortunately, the dosage often refers 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 approx. 6 and 12 for rat and mouse respectively, in comparison to man (for review see Morris, 1995). By analogy, the half-life time of therapeutics is in general reduced in small rodents requiring more frequent application for maintaining an effective level of the therapeutic.

The treatment of parasitic invasions is in particular dependent on the accompanying hygienic procedures, e.g. use of gloves, chemical and/or physical disinfection of the animal rooms, cages, lids, bottles. In Table 3 some commonly used antiparasitics are summarized. For additional drug dosages, see Hawk and Leary (1995). In the case of parasitic eggs and oocysts, a chlorcresol[1] formulation has proven particularly valuable. It should be mentioned, however, that treatment may be associated with toxic effects (Scopets *et al.*, 1996; Toth *et al.*, 2000). Ivermectin induced long-lasting alterations, particularly in bone marrow derived macrophages (Mossmann and Modolell, unpublished).

Chemotherapeutic and antibiotic treatment of infections may induce resistance, especially when used on a large scale, on growth of other bacterial species (Hansen, 1995), adverse reactions by shifting the gut flora (for review: Morris, 1995), or derangements of physiological functions (el Ayadi and Errami, 1999). Commonly recommendable treatment procedures of infected animals are given in Table 4.

1 Chlorcresol (Neopredisan): Menno-Chemie Vertrieb GmbH, D-22850 Norderstedt.

Table 3. Treatment of common parasites (in combination with hygienic measures)

Generic name	Trade name	Application	Dose	Reference
Ectoparasites				
Ivermectin	Ivomec*	Topical spray	0.2–10 mg^{-1}	Hirsjärvi and Phyälä (1995)
Endoparasites[†]				
Piperazine citrate	Piperazin*	Drinking water (for 12 weeks, every 2nd week)	0.2%	Maess and Kunstyr (1981)
Fenbendazole[‡]	Panacur* Coglazol*	Diet several months	150 ppm in diet or 8–12 mg/kg/day	Coghlan *et al.* (1993) Huerkamp *et al.* (2000) Wilkerson *et al.* (2001)
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) Zenner (1998)

* Pharmazeutische Handelsgesellschaft, Siemensstr. 14, 30827 Garbsen.

[†] Especially *Syphacia obvelata* and *Aspiculuris tetraptera*.

[‡] Diet can be autoclaved without substantial loss of efficacy.

Table 4. 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) Macy *et al.* (2000)
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 (*Heliobacter* sp.[†])	Amoxillin[‡]	For 4 weeks – young mice	50 mg kg^{-1}	Russel *et al.* (1995)
	Amoxicillin[‡] Metronidozole Bismuth	For 2 weeks	200 mg l^{-1} 138 mg l^{-1} 37 mg l^{-1}	Foltz *et al.* (1996)
Pneumocystosis/mouse, rat	Sulfadoxine–trimethoprim (Borgal; Trimethosel, Cotrim K)	For 3 weeks	200 mg g^{-1} l^{-1}	H.-J. Hedrich (unpublished)
Tyzzer's disease (*Clostridium piliforme*)/ mouse, rat, rabbit	No antibiotic therapy recommended; derivation Special disinfectant required[§]			

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

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

[†] Especially immunodeficient mice.

[‡] Toxic for hamster and guinea-pigs.

[§] Chlorocresol (Neopredisan).

Infection experiments

General precautions

The safe operation of an animal laboratory is one of the main management responsibilities. Housing infected animals require 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 few and seldom found 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 infectious 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 aiming at prevention of infections for laboratory personnel. Many programmes were developed in response to evaluations of laboratory accidents. Most laboratories have written control plans, which 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 animal facilities housing animals behind barriers. Only major points can be discussed here; more details on general laboratory safety are given in many textbooks on clinical microbiology (Burkhardt, 1992; Strain and Gröschel, 1995) and in general recommendations for housing of laboratory animals (CCAC, 1980; Kunstyr, 1988b; Bruhin, 1989; BG Chemie, 1990; National Research Council, 1996, 1997; Smith, 1999).

Education is an important part of effective safety programmes. 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, like the use of personal protective clothes. Prohibition of eating, drinking, smoking, handling of contact lenses and the application of cosmetics in the laboratory are other basic rules, like 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 for 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 on mucous membranes are mouth pipetting and hand–mucosa contact. Both must be strictly forbidden.

Microisolator cages are often used in animal facilities for transportation within the facility 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 for humans or other animals. Experiments with infectious agents will usually be conducted in separate areas which fulfil all safety requirements like 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 properties of the agents like pathogenicity, environmental stability, or 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 better suited than microisolators to prevent spreading of micro-organisms if they are properly handled. 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, which can be locked 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 as to whether or not waste from animals that are not experimentally infected is infectious 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 is dependent 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 reduce the infectious risk 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.

Biosafety for housing laboratory animals

Biosafety criteria for housing vertebrates have been defined in the USA by CDC (1988) for biosafety levels 2 and 3 and later for all four biosafety levels (CDC/NIH 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 the laboratories and are managed under the responsibility of a research director. Large research institutions, companies or universities often have centralized laboratory animal facilities, which are managed by laboratory animal specialists. They are usually separated from laboratories or institutes. Such facilities usually fulfil more easily 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–long term) for different scientific disciplines (e.g. toxicology, immunology, biochemistry). Several housing systems (conventional units, barrier units, isolators) or microbiological quality standards (infected, pathogen-free, gnotobiotic) can be found in large facilities. 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 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 facilitates proper cleaning and personal hygiene. Bedding material from animal cages is removed in a manner that avoids the formation of dust or aerosols and minimizes the risk of allergies, thus reducing 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 such 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 activities of the animals themselves can introduce new hazards

by producing dust or aerosols, or they may traumatize humans by biting and scratching. Therefore, CDC/NIH (1993) established standards for activities involving infected animals which are designed 'animal biosafety levels' (ABSL) 1–4. These combinations describe animal facilities and practices applicable to work on animals infected with agents assigned to corresponding BL-1–4.

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; National Research Council, 1996, 1997). In contrast to experiments with non-infectious materials, additional hygienic 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 (best 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 the 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 a ventilated cage 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.

As with BL-3 materials, access to an ABSL-3 facility is very much restricted. All 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 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 carried out in closed containers thus reliably avoiding a risk of transmission. Very few facilities house ABSL-3 animals. If this is really necessary, many more safety precautions will be taken than recommended by CDC/NIH (1993) (e.g. a one-piece positive-pressure suit that is 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 can take place by scratching or biting. A maximum of access control and of hygienic measures are necessary.

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