Lessons from gene knockouts

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Summary
The authors describe the technique for the application of homologous recombination in embryonic stem cells, which is now widely used to engineer mice which carry specific knockouts of genes. A summary is given of some of the knowledge of the pathogenesis of and resistance to infections with parasites, bacteria, or viruses which has accumulated during recent years, based on the investigation of knockout mice. Special emphasis is placed on knockout animals which lack components of the cytokine network, lack genes which are critical for the correct presentation of antigens or are deficient in different immune cell subsets. In addition, a brief explanation is offered of the possibilities for inducing targeted deletions or mutations in genes of livestock species (e.g., by nuclear transfer or by mutagenesis using the alkylating agent N-ethyl-N-nitrosourea) which could lead to the breeding of animals which are resistant to infectious diseases in the future.

Keywords

Gene targeting in embryonic stem cells: a powerful tool for analysing gene function

Embryonic stem (ES) cells are pluripotent cell lines which are reproducibly derived from the inner cell mass of mouse blastocysts (23, 58). Differentiation of ES cells is inhibited by leukaemia inhibitory factor (LIF) or other cytokines of the interleukin-6 family which allow long-term culture and genetic manipulation of ES cells in vitro (100, 101, 102). Upon injection into blastocysts (12) or aggregation with morula stage embryos (103), ES cells may contribute to all tissues of the resulting chimera, including its germ line (Fig. 1). Therefore, the possibility exists – at least indirectly – to reconstitute mice from cultured cells. The need to produce chimeras can be circumvented by aggregating ES cells with tetraploid embryos (62). Using this technique, researchers have obtained completely ES cell-derived offspring: however, these were not fertile and thus not suitable to establish transgenic lines.

ES cell technology is now widely established and offers a powerful tool for functional analysis of genes. Based on the principle of homologous recombination (19), various strategies have been developed to introduce targeted mutations into ES cells (36) (Fig. 2). Although the efficiency of homologous recombination was low in early experiments, the frequency of targeted integration was markedly increased by using isogenic DNA for the construction of targeting vectors (87).

Initial studies were directed principally towards a complete inactivation of gene function to evaluate the biological relevance of the respective genes (13, 14). The production and use of these so-called 'gene knockouts' and the corresponding knockout mice are now standard techniques in many laboratories. In the past few years, new methods have widened the spectrum of ES cell technologies by allowing the introduction of subtle site-directed mutations, large chromosomal alterations and tissue-specific gene knockout and repair. This allows analysis of gene function in a lineage-specific way and also permits the analysis of genes which lead to embryonic lethality if inactivated in early embryonic development (69). The most widely used system
Fig. 1  Schematic illustration of the production of germ-line chimeric mice by morula aggregation or blastocyst injection

for introducing these fine-tuned mutations in mice is the Cre/lox-P system from bacteriophage P1 (44).

This repertoire of methods has been used extensively to dissect the functions of genes involved in complex biological systems, including those relevant for the immune system and for resistance or susceptibility to infectious diseases.

Targets for gene disruption to investigate the pathogenesis of infectious diseases

To investigate the molecular pathogenesis of infectious diseases and combat infectious diseases by breeding measures, two different approaches can be followed. In the first approach, the aim of generating transgenic or knockout animals can be to prevent attachment, entry or replication of the infectious agent into the respective hosts. The most striking example of this approach is the generation of PrP\textsuperscript{\textminus} knockout mice which are resistant to infection with the scrapie agent (17). With the second approach, the immune response of the host to different pathogens can be investigated by interfering with the immune system and by targeted disruption or modification of genes which are involved in immune mechanisms (Fig. 3).

Fig. 2  Strategies for introducing targeted mutation into the genome of embryonic stem cells

The thick line represents the vector homology to the target locus; the broken line represents the bacterial plasmid. The hatched rectangles are exons. The positive selection marker is the neomycin phosphotransferase (neo) gene. The thymidine kinase (TK) gene of herpes simplex virus is used as a negative selection marker

a) Replacement vector: the positive selection marker interrupts the target homology

b) Insertion vector: a positive selectable marker may be cloned into the homologous sequences or the vector backbone. A double strand break is generated in the target homology prior to transfection

Fig. 3  Possible target genes for the generation of knockout animals to study the pathogenesis of infectious diseases

To date, few reports and applications have dealt with the prevention of entry of infectious agents into knockout animals. This is largely due to the fact that the molecular mechanisms involved in the early stages of infection of most infectious pathogens remain unclear, and the lack of information on these processes prevents the targeted disruption of genes and gene products which would prevent initial infection. However, as knowledge about the immune system in man and animals has increased significantly during the last years, the interaction of individual components of the immune system, both on the level of the immune cells and the soluble mediators which regulate the immune cascade (cytokines and chemokines, as well as cytokine and chemokine receptors) with pathogens was addressed using knockout technology. Figure 4 gives a schematic overview of...
the current knowledge of the immune system and the regulatory and modifying mechanisms underlying the defence against infectious agents. As different infectious agents are encountered by different immune measures of the host, the first goal of targeted inactivation of genes is to determine which factors in the complicated immune network have essential, beneficial or detrimental effects on the capacity of the host to control an infectious agent and the disease caused by the pathogen. Virtually all molecules involved in the immune network can be a target of gene disruptions. However, depletion of single chemokines or chemokine receptors, for example, may cause a general breakdown of the immune system, might be compensated by other related mechanisms, or may have specific effects on individual infectious pathogens (47). The following description of selected reports represents examples of knockout animals which were used to address specific questions of the interaction of the host with infectious agents.

Lessons from knockouts: the mouse as a model

As stated above, considerable progress has been made during recent years in identifying mammalian genes and gene products that influence the outcome of disease after infection with different agents, including parasites, bacteria and viruses. By using knockout technology, a better understanding of the cytokine activities upon infection and of the interactions of individual cytokines during acute or chronic infectious disease has been obtained, at least in widely used mouse models of infectious diseases (see below and Table I). In addition, a large number of regulatory pathways underlying the maturation of immune cells and their direction to the active sites of the immune defence has been elucidated (25, 40).

One of the most important issues in using knockout models is the understanding of the genetic basis for resistance or susceptibility to infectious diseases. This has become an issue of major interest in disease control and for the rational design of novel vaccine approaches, which are extremely important in a time of increasing numbers of immunocompromised individuals. Hence, knockout models are used to more precisely define:

- the mechanisms underlying acute and chronic disease
- the clearance of the pathogen from the organism
- immunity to infection or at least disease.

In acknowledging and using the great advantages of transgenic and knockout models, however, it must be also noted that pathogenesis and immunity models of animal or human infectious diseases which rely on the use of knockout animals must be substantiated by other experimental procedures. This restriction of transgenic or knockout animals is caused by the existence of subtle feedback mechanisms in the organism which are influenced by targeted gene
disruption or expression, and also by the nature of any knockout and transgenic models which are very contrived per se (9, 54). The second important restriction relates to the fact that the information obtained is so far mainly restricted to the mouse, which is used as a model for many infectious diseases.

The next paragraphs list some of the recent findings on the outcome of disease after infection experiments with different pathogens which have been elucidated using knockout mice. The presented data will mainly concentrate on protozoan, bacterial and especially virus diseases.

### Resistance to infection with parasites

Parasitic infections have become a major issue world-wide. This is partially - though not exclusively - caused by the growing number of individuals who suffer from immunosuppressions. Of particular importance in the generation of an immunocompromised state in man are infections with human immunodeficiency virus type 1 (HIV-1) and also allergic diseases, whereas in animals there is a variety of causes of immunocompromised individuals. Human infections with different protozoan pathogens such as Eimeria spp., Plasmodium spp., Leishmania major, Trypanosoma spp., Toxoplasma gondii or Pneumocystis carinii are also of great concern.

Research which applies the use of knockout animals to investigate the outcome of disease with these pathogens has concentrated on the beneficial or harmful effects of cytokine action. An infection with T. gondii can cause an acute or latent infection. Acute infections are caused by rapidly dividing tachyzoites which harm virtually every cell of the body, whereas latent infection is characterised by dormant bradyzoite forms in tissue cysts (30). With regard to the molecular pathogenesis of T. gondii infection, there has been speculation that infection is controlled primarily by cell-mediated immune responses and interferon-γ (IFN-γ) activity (82). The effect of IFN-γ was thought to be augmented by the effects of interleukin-12 (IL-12) (32). By using IFN-γ knockout mice, Scharton-Kersten et al. have shown, however, that parasite control depends on IFN-γ alone and that in the absence of the cytokine, tachyzoite replication is unlimited (73, 74, 75). In contrast, IL-12 was produced to levels found in wild-type mice, thus demonstrating an indirect function of IL-12 through IFN-γ in control of T. gondii infection, since the infection could not be

### Table I

Examples of mice with targeted deletions of cytokine or cytokine receptor (R) genes, and infected with parasites, bacteria or viruses

<table>
<thead>
<tr>
<th>Targeted deletion</th>
<th>Treated/infected with</th>
<th>Comments</th>
<th>References</th>
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<tbody>
<tr>
<td>IL-1β</td>
<td>Lipopolysaccharide</td>
<td>Lower body temperature</td>
<td>24, 50</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Influenza virus</td>
<td>Higher mortality</td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>Escherichia coli</td>
<td>Partial resistance to challenge</td>
<td>1</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Lipopolysaccharide</td>
<td>Resistance to challenge</td>
<td>1</td>
</tr>
<tr>
<td>IL-2</td>
<td></td>
<td>Breakdown of self-tolerance</td>
<td>40</td>
</tr>
<tr>
<td>IL-3R</td>
<td>Nippostrongylus brasiliensis</td>
<td>Effect on GM-CSF and IL-5; reduced eosinophilic counts</td>
<td>63</td>
</tr>
<tr>
<td>IL-4</td>
<td>Toxoplasmoid gondii</td>
<td>Higher mortality rates</td>
<td>87</td>
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<tr>
<td>IL-4</td>
<td>Leishmania donovani/L. mexicana</td>
<td>Different disease progression</td>
<td>72</td>
</tr>
<tr>
<td>IL-4</td>
<td>Plasmodium chabaudi</td>
<td>Reduced Th2-associated cytokines</td>
<td>93, 94</td>
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<tr>
<td>IL-4</td>
<td>Trypanosoma brucei brucei</td>
<td>Strain-dependent higher parasitemia</td>
<td>3, 4</td>
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<tr>
<td>IL-5R</td>
<td>See IL-3R</td>
<td></td>
<td></td>
</tr>
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<td>IL-10</td>
<td>Trypanosoma cruzi</td>
<td>Lower parasite burden but earlier mortality</td>
<td>42</td>
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<tr>
<td>IL-10</td>
<td>T. gondii</td>
<td>Lethal immune response</td>
<td>33</td>
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<td>TNF-α</td>
<td>Listeria monocytogenes</td>
<td>Impaired resistance and clearance</td>
<td>85</td>
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<td>TNF-αR</td>
<td>E. coli</td>
<td>Mild effect</td>
<td>1</td>
</tr>
<tr>
<td>TNF-βR</td>
<td>L. monocytogenes</td>
<td>Impaired resistance and clearance</td>
<td>85</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>T. gondii</td>
<td>Earlier mortality</td>
<td>73, 74</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>L. donovani</td>
<td>Uncontrolled visceral infection</td>
<td>86</td>
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<tr>
<td>IFN-γ</td>
<td>Legionella pneumophila</td>
<td>Development of persistent disease</td>
<td>38</td>
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<tr>
<td>IFN-γ</td>
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<td>IFN-γ</td>
<td>Lymphocytic choriomeningitis virus</td>
<td>No virus clearance in persistently infected mice</td>
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<td>IFN-γ</td>
<td>Plasmodium berghei</td>
<td>No cerebral malaria</td>
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<td>L. monocytogenes</td>
<td>Higher IL-4 production and susceptibility to disease</td>
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<tr>
<td>IFN-γ</td>
<td>Influenza virus</td>
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IL : interleukin
TNF : tumour necrosis factor
IFN : interferon
GM-CSF : granulocyte macrophage colony-stimulating factor
Th2 : T helper 2

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controlled by IL-12 alone. Mice which no longer expressed the IFN-γ regulatory factor 1 (IRF-1) gene and which, as a consequence, were unable to produce nitric oxide (NO), the potent inactivator of intracellular (macrophage) parasites, were more susceptible to infection, although NO was not exclusively responsible for clearance of the pathogen (48). In mice which were devoid of another cytokine, IL-10, T. gondii infection, instantly caused high mortality which was characterised by increased levels of IFN-γ and IL-12. In addition, other key cytokines, such as IL-1β and tumour necrosis factor α (TNF-α) were augmented, which suggests that the modifying cytokine IL-10 regulates other cytokines and that in the absence of IL-10, IFN-γ and IL-12 cause a CD4+ (cluster of differentiation antigen) T-cell-dependent immunopathological disease (33). These findings were substantiated by studies which analysed IL-4-deficient mice and their susceptibility to T. gondii infection (67). IL-4 knockout mice produced significantly lower levels of IL-10 but higher levels of IFN-γ, and mortality rates were greatly enhanced.

Infections of animals or man with Trypanosoma cruzi (Chagas' disease) and T. brucei lead to disturbance of the host immune response and are characterised by an unspecific activation of the immune system, which finally results in autoimmune reactions and pathological consequences (15, 35). Resistance to disease has long been known to depend on IL-10 production and its suppression of IFN-γ production, and infection is known to be aggravated by IFN-γ (4, 60). Using mice which were deficient in IL-10, infection with T. cruzi could be shown to lead to earlier and higher mortality, although the parasite burden was lower when compared with wild-type mice (42). The same observation was made after infection of mice lacking γδ T cells (a lymphocyte subclass which is stimulated by IFN-γ and downregulated by application of IL-10) (52), or by using mice which were deficient in CD4+ or CD8+ cells (85). These results suggest a complex interaction between immune cells in the control of infection and their interdependence in parasite clearance and disease outcome. IFN-γ knockout mice displayed higher resistance to T. brucei infection (4), thus indicating that the regulation of and mediation by this key cytokine (Th1 helper cell activation) plays a dominant role in susceptibility and resistance to infection with Trypanosoma spp.

Other parasites of growing importance are L. donovani and L. major. These parasites cause leishmaniosis, a proliferative and necrotic disease of the skin and inner organs which is controlled primarily by the action of IFN-γ and by a T helper 1 (Th1) type immune response (61, 66, 80). The knowledge of Leishmania pathogenesis and the control of infection by different types of immune cells and cytokines is largely based on the use of knockout animal models and subsequent infection experiments. In the absence of Th1 cells and IFN-γ, macrophages which are responsible for parasite clearance are not activated and protozoan replication is unrestricted (55, 96, 97). Recent work, however, demonstrated that L major pathogenesis is not enhanced if major histocompatibility complex (MHC) class II presentation is hampered in mice that lack the invariant chain. Obviously, cytokines such as IL-12 and IL-4 were able to induce maturation of T cells in the absence of functional MHC class II molecules, such that IFN-γ is secreted (16). These results were confirmed and extended by the use of IFN-γ knockout mice in which a TNF-α-dependent action of IL-12 in the control of leishmaniosis could be demonstrated (86).

Experiments comparable to those described above on the role of γδ T cells for T. cruzi have been performed with mice lacking different immune cell subsets using the protozoan parasites Eimeria spp. and Plasmodium spp. Mice which no longer expressed either αβ or γδ T cells exhibited increased susceptibility to intestinal infection with Eimeria spp., although this appears to be due to different mechanisms: the lack of αβ+ T cells resulted in a reduced immunity to reinfection, whereas lack of γδ+ T cells led to enhanced intestinal damage (68). As a result of B-cell depletion by knockout technology, enhanced resistance to acute Plasmodium spp. infection could be demonstrated. This appears to be due to a rise in γδ+ T cells. In addition, a rise in αβ+ T cells could be demonstrated in B-cell-deficient animals, although this rise was not as marked as that in the γδ subset (91). The authors conclude that this T-cell subset is largely controlled by B-cell action, that this activation is independent of IL-10 production, and that the expansion of γδ T cells might be a target for further vaccine strategies which are able to stimulate specifically this T-cell subset.

Taken together, the reports summarised above have largely improved the understanding of protozoan infection, and have revealed a complex concert of cytokines in control of resistance to and progression of infection. Thus, these findings will no doubt be useful in future attempts to generate a new generation of anti-parasitic drugs and efficacious vaccines.

**Resistance to disease caused by bacteria**

By generating transgenic and knockout animals, the pathogenesis of a variety of bacterial diseases has been elucidated. The role of B- and T-cell subsets and/or individual cytokines involved in the resistance or susceptibility to bacterial disease has been assessed, and special emphasis was put on the molecular mechanisms underlying sepsis and clearance of intracellular bacteria.

Sepsis caused by gram-negative bacteria is one of the most important causes of mortality after surgical intervention, and is caused by a shock syndrome which follows lipopolysaccharide (LPS) or endotoxin release (10, 21). A critical step in the development of LPS-caused shock is the release of the key cytokines TNF-α and IL-1β, which cause the attraction and stimulation of immune cells locally in the micro-environment. However, the same cytokines are able to induce responses which are indicative of septic shock, not in their paracrine but in their systemic functions (20, 27). The
resistance to TNF-α- and IL-1β-induced shock was tested after application of *Escherichia coli* or endotoxin in respective knockout mice. Animals deficient in either cytokine were shown to exhibit increased resistance to intravenous application of endotoxin, thus indicating that acute and systemic reaction and activation of immune T cells is suppressed. In addition, in the absence of IL-1β, LPS-induced fever is no longer observed. Hence, this mechanism plays a major role in disease progression (1, 50). Similarly, mice deficient in CD14 (which serves as a receptor for LPS) are much more resistant to the shock syndrome caused by gram-negative bacteria, as shown in an infection model. In addition, CD14-negative mice exhibited reduced levels of bacteraemia, which suggests that the spread of gram-negative bacteria at least is controlled by a CD14-dependent mechanism (37). The knowledge of the early interaction of gram-negative bacteria with the host immune response could be beneficial in the treatment or immune prophylaxis against these pathogens, which are a frequent cause of mortalities in the human and animal populations.

Most other reports on the genetic resistance to bacterial infections have dealt with organisms which replicate intracellularly or which interfere with host cells other than by endo- or exotoxin production. Among the bacterial infections which were analysed using knockout animals are those with *Listeria* spp., *Brucella* spp., *Mycobacterium* spp. or Francisella *tularensis*. The deletion of the β2-microglobulin gene leading to an absence of a functional CD8-dependent cytotoxicity, and also the deletion of functional CD4+ cells, were shown to cause delayed clearance of both *Listeria monocytogenes* and *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) from infected animals. However, infection with either bacterium was not fatal in these animals, thus suggesting a certain redundancy in the host response to the pathogens (46, 47). The disruption of the IFN-γ receptor gene led to decreased levels of TNF-α, and *Listeria* infection was more severe when compared with that in control mice. Obviously IL-4, which is not measurable after *L. monocytogenes* infection in wild-type mice but which is produced by mice which lack the IFN-γ receptor, is responsible for the more severe symptoms. In contrast, IL-12 did not appear to be involved in *Listeria*-caused disease (83). Using mice which are deficient in the macrophage type I and II class A scavenger receptors (proteins which are involved in binding of bacteria), disease after infection with *L. monocytogenes* was shown to be augmented, which demonstrates that receptor-mediated incorporation and presentation of the antigens by macrophages is essential in the host defence against intracellular pathogens (81).

In similar animal models (i.e., using mice with MHC class I or II knockouts), researchers were able to demonstrate that *Brucella abortus* clearance and resistance to disease is caused mainly by the cytotoxicity of CD8+ T cells (79). By identifying the host effectors and the targets in the bacterium, the authors intend to produce rationally designed novel vaccines against this zoonotic pathogen. Using animals which are deficient in different T-cell subsets, the resistance and immunity to *F. tularensis* infection was analysed. The antibacterial response was shown to be based mainly on αβ T cells, whereas depletion of CD4+ cells or γδ T cells did not alter disease control appreciably. The authors concluded that in general, resistance to intracellular bacteria is largely dependent on the αβ T-cell subset, but that CD4+ or CD8+ cells can control the disease alone and independently (105).

IFN-γ knockout mice have been used to analyse the outcome of infection with *Legionella pneumophila*. These experiments demonstrated that IFN-γ action is essential for clearance and control of pulmonary infection with *Legionella*. The antimicrobial activity of IFN-γ after infection with this pathogen appears to be dependent on the synthesis of the inducible nitric oxide (NO) synthetase, which is upregulated by IFN-γ and which is obviously responsible for intracellular killing of the bacterium which prevents systemic spread of *Legionella* to virtually all tissues (38).

**Resistance to virus infection and disease**

Recently, intensive research concerning the resistance to infection or disease caused by infectious pathogens has been performed with viruses. This is probably caused by the nature of the pathogens which – due to their relatively small genomes – are well characterised on a molecular basis. As a consequence, unlike the situation with more complex organisms (e.g., parasites or bacteria), a pathogen-caused effect can often be reduced to one or few viral gene products, and thus the ‘choice’ of transgenic or knockout animal models is facilitated. A large number of reports on genetic resistance to viral disease have been based on the analysis of knockout animals: a small and non-representative collection of this research will be presented below.

Most reports concerning the effect of targeted knockouts and their influence on the outcome of disease deal with the role of different immune cell subsets, of antigen presentation by either MHC class I or II, and of individual cytokines as described above for protozoan and bacterial infections. The use of CD4+/− and CD8+/− mice and infection with influenza viruses demonstrated that neither cell type appears to be required for the clearance of the virus at the effector stage (22). In addition, the same authors could demonstrate by the use of J-chain knockout mice (which are no longer capable of forming secretory multimeric immunoglobulin A [IgA]) that immunity to influenza is not dependent on the presence of secretory IgA. Mice lacking mature B cells after the deletion of the Ig μ chain also did not alter appreciably the efficiency of immune responses to influenza viruses, or the generation and maintenance of helper T cells (90). In contrast, mice which are deficient in the key cytokine IL-1 exhibited higher mortality rates after infection, which were apparently caused by the inability of these animals to develop fever (50).
Arenaviridae, various reports on the use of knockout animals in contrast to the clearance of acute virus loads - which was at least partially caused by the absence of cytotoxic T lymphocyte (CTL) memory cells (59, 88). In the context of the co-operative effect of different immune cells in LCMV control, the interaction of CD40 and CD40 ligand (CD40L) was addressed by the use of CD40- or CD40L-deficient animals. The results demonstrated that the CD40-CD40L interaction is important in the interaction between B and T cells, but is far less important in T-cell activation. This is due to a failure of CD40- and CD40L-deficient animals to perform a switch in Ig isotypes and to generate antibody to T-cell-dependent antigens (64). Furthermore, the presence of CD8+ T cells is absolutely critical in both the acute and chronic clearance of LCMV infection, as has been demonstrated by the use of β2-microglobulin knockout mice (5, 59). This higher susceptibility of animals to LCMV is dependent on the production of IFN-γ and subsequent virus clearance by the induced CD8+ cytotoxic T cells (89). These results are supported by the findings of Walsh et al., who showed the inability of perforin-deficient mice to clear LCMV because they are unable to eliminate infected cells. This inability to clear LCMV-infected cells is even potentiated in the absence of FAS-mediated apoptosis (56, 95). Thus, two complementary cytotoxic mechanisms are used in the elimination of LCMV from infected cells and for the resistance to particularly long-term infection.

An important infection of neonates is rotavirus diarrhoea, which causes damage to the gastrointestinal tract. However, immunity to rotaviruses is poorly understood. By using β2-microglobulin knockout mice, researchers demonstrated that acute virus infection can be cleared in the absence of CTL responses and that CTL are not necessary for the development of a sustainable immunity. In contrast, as shown by the use of B-cell-deficient mice, immune B cells play a major role (probably by the generation of IgA) in the clearance of acute rotavirus infection and longevity of immunity (28). Recently, these findings were corroborated by the same authors and defined more precisely: using perforin-, FAS-, and IFN-γ-deficient mice, no increased susceptibility to rotavirus infection was observed, although CD8+ T cells appear to be involved in virus clearance from the body by a mechanism that is independent from their cytotoxic activity (29).

Coxsackie viruses, which are members of the genus Enterovirus of the family Picornaviridae, can cause fatal heart disease, especially in immunocompromised individuals, as a result of acute or chronic myocarditis. The question of whether direct virus effects or immunopathological processes cause the diseases associated with the virus is unresolved. Knockout mice which were deficient in CD4+ cells or β2-microglobulin were shown to be less susceptible to acute disease. In addition, it could be clearly demonstrated that, although high virus titres in mouse hearts post infection were observed after CD8+ cell depletion, these mice recovered earlier from infection and death rates were markedly reduced (39): the same results were seen in mice with a targeted deletion of the T-cell tyrosine kinase (53). Thus, Coxsackie virus damage to the heart appears to be largely dependent on immunopathological mechanisms, although following knockout of the IFN-γ receptor, a high susceptibility to acute infection, which is caused by the absence of any inducible NO synthetase – a prerequisite for clearance of intracellular pathogens, especially in antigen-presenting cells – could be observed (53).

Some information on HIV-1 pathogenesis and immunity has been obtained using knockout mice and murine leukaemia viruses as a model virus-host system for human infection. In the absence of B cells, initial virus replication was diminished. This reduction in virus replication is probably due to the absence of the primary target cells, which consequently leads to a reduced virus burden and T-cell and macrophage infection (49, 76). Further work identified CD8+ T cells as the main effector cells in the resistance to mouse acquired immune deficiency syndrome (AIDS), which is at least partially dependent on perforin function. However, in both β2-microglobulin and perforin knockout mice, no progression to lymphoproliferation was observed, thus suggesting that other mechanisms besides CD8+ T-cell-dependent cytotoxicity are involved in resistance of the animals to the retrovirus (84). Recently, the myeloencephalopathy which is caused by murine leukaemia virus, and which is characterised by a paralysis and neurodegenerative disease indistinguishable from that caused by prions, has been shown to be independent of the absence or presence of the PrP C protein. These results suggest that, although clinically and histologically similar diseases are observed after infection with either prions or murine leukaemia virus, the two agents act in different ways. Some scientists have speculated that murine leukaemia virus might use the same pathway as prions but interfere at a point downstream of the PrP C protein.

A relatively large number of reports on the use of knockout animals deals with the pathogenesis of and immunity to herpes simplex virus type 1 (HSV-1). Despite the intensive and thorough characterisation of this virus, the pathogenesis of herpetic lesions, latency and the short duration of immunity are less well defined. Using knockout animals and a murine model of HSV-1 infection, some revelations on the cytokines and the subsets of immune cells which are involved...
in the control of infection have been obtained. Results demonstrated that in the absence of CD4+ cells – in contrast to the absence of CD8+ cells – a marked increase in susceptibility to herpetic lesions was evident (57). These results confirmed and extended previous observations that cytotoxic CD4+ cells are primarily responsible for the control of HSV-1, especially in neuronal tissues. However, the absence of IFN-γ, which was thought to play a major role in the pathogenesis of ocular lesions, did not alter the disease appreciably. The findings showed that IFN-γ is involved in virus clearance but that other cytokines and immune responses are also functionally active in HSV-1-caused disease (11, 106). Nonetheless, IFN-γ-deficient mice were able to develop sustainable anti-HSV-1 immune responses (106), which agrees with the complex cascades that orchestrate anti-HSV-1 immunity.

Although the use of knockout animals in carcinogenesis studies is widely accepted and has improved the understanding of different effector and immune mechanisms, few reports on virus-induced cancer and the use of knockout models are available. Mouse mammary tumour virus (MMTV)-ras transgenic animals which lack the tumour suppressor p53 were affected with tumours earlier than control mice. Using this model, it could be demonstrated that p53 can act in an apoptosis-independent manner in the elimination of tumour cells (41). The absence of CD4+ or CD8+ cells led to a higher incidence of polyomavirus-induced tumours, which was even higher in CD4+/CD8+ double knockout animals (7, 8), thus indicating that both classes of immune T cells play important and interdependent roles in the control of virus infection and tumourigenesis. By using different knockout animals and models of tumour induction by viruses or viral gene products, several mechanisms underlying the progression of tumour cells or their elimination have been elucidated. Tumourigenesis has been shown to be essentially dependent on cell cycle progression and on the failure of apoptotic mechanisms induced by several independent pathways. In addition, the suspected involvement of the double-stranded RNA-dependent protein kinase induced by type I interferons in tumour suppression could not be confirmed (77, 78, 104).

**Targeted mutagenesis of genes in species other than the mouse**

Although a number of ES cell-like cell lines have been reported in species other than the mouse, successful germ-line transmission of these cells has not been proven for any of these species (2, 31). However, recent progress in cloning animals by nuclear transfer offers new strategies for targeted mutagenesis of genes in livestock species. Potential practical uses of nuclear transfer in livestock species include both the multiplication of embryos produced from selected matings and the ability to produce offspring from cells which can be maintained in culture prior to embryo reconstruction, thereby providing a route for precise genetic modification.

The latter approach appears to be feasible in the future, as live offspring have been obtained from sheep following nuclear transfer from cell lines of embryonic (18, 98), foetal and even adult origin (99) (Fig. 5). Whether this technology can be transferred to other species remains to be shown; however, successful cloning in calves using cultured cells from the inner cell mass of blastocysts as nuclear donors seems promising (26). These attempts may provide the possibility to generate livestock with resistance to different diseases.
Complementary approaches for the discovery of genes relevant for infectious diseases

The most important tool to obtain insight into the biological function of genes is the use of mutants. The use of ES cells and homologous recombination allow the systematic production of mouse mutants for any gene which has been cloned. Complementary to such a 'gene-driven' approach, 'phenotype-driven' approaches will be necessary to identify new genes or gene products through a search for new mutants with specific defects, for example in immune function and resistance to infectious diseases.

Mutagenesis of mice using the alkylation agent ethynitrosourea (ENU) is a powerful approach for the systematic production of mouse mutants for a number of reasons, as follows (6):

- Protocols are available which allow a very efficient mutagenesis rate in mice. The frequency of mutant recovery is about 1/1,000 for a specific locus which can be scored phenotypically, although strain as well as dosage and treatment regimens do influence the mutagenesis rate.
- ENU mutagenises premeiotic spermatogonia of F₀ males which can be bred to produce a large number of F₁ offspring or F₃ pedigrees, respectively.
- ENU induces mainly point mutations. The mutants produced will therefore be mainly hypomorphic, although gain-of-function and complete loss-of-function mutants can also be expected.

The systematic production and analysis of ENU mutants involves a number of different steps (Fig. 6). F₀ males of an inbred strain are treated with ENU and, after a transient period of sterility, are bred with females of the same genetic background to produce F₁ offspring and subsequently F₃ pedigrees. F₁ and F₃ mice are scored phenotypically for dominant and recessive mutations, respectively. Once a mutant has been confirmed phenotypically and genetically, the chromosomal location of the mutation can be mapped using mice from an outcross/backcross panel produced with another inbred strain by genome-wide microsatellite-typing of 'mice showing versus mice not showing' the particular phenotype. Mutations induced by ENU will not be tagged molecularly. Although this is initially a disadvantage with respect to the cloning of the responsible genes, the availability of point mutations will be very important for a more detailed functional analysis. Furthermore, the advances that are currently made in the field of genomics, particularly the production of high resolution genetic, physical and transcript maps will reduce the difficulties inherent in the cloning of the genes mutated after ENU treatment.
Les leçons de l’invalidation de gènes

N. Osterrieder & E. Wolf

Résumé
Les auteurs décrivent la technique de recombinaison homologue appliquée aux cellules souches embryonnaires, très utilisée aujourd’hui pour obtenir des souris « knockout » dont certains gènes spécifiques ont été éliminés. Ils présentent une synthèse des connaissances sur la pathogénie des infections parasitaires, bactériennes ou virales, qui se sont multipliées ces dernières années, à partir de recherches effectuées avec des souris « knockout », et sur la résistance à ces infections. Ils mettent notamment l’accent sur les animaux « knockout » dépourvus de certaines composantes du réseau des cytokines et de certains gènes importants pour une bonne présentation des antigènes ou dont les différentes sous-populations de cellules du système immunitaire présentent des déficiences. Les auteurs présentent également un bref exposé sur les possibilités d’induire des délétions ou des mutations ciblées dans les gènes d’espèces animales (par exemple, par transfert nucléaire ou par mutagenèse à l’aide de l’alkylant N-ethyl-N-nitrosourée) qui pourraient permettre de sélectionner, à l’avenir, des animaux génétiquement résistants aux maladies infectieuses.

Mots-clés

Enseñanzas de las supresiones específicas de genes (gene knockouts)

N. Osterrieder & E. Wolf

Resumen
Los autores describen la técnica para aplicar la recombinación homóloga en células primordiales embrionarias, actualmente muy utilizada en ingeniería genética para crear ratones portadores de determinadas supresiones específicas (knockouts) de genes. El artículo contiene un resumen de los conocimientos sobre la patogénesis de las infecciones parasitarias, bacterianas o viricas y la resistencia a tales infecciones que han ido deparando en los últimos años las investigaciones con ratones mutantes (ratones knockout). Se hace especial hincapié en los mutantes desprovistos de algún componente de la red de citocinas o de genes cruciales para la correcta presentación de antígenos, y en los que son deficientes en algunas subclases de células del sistema inmunitario. Se comentan además sucintamente las posibilidades de inducir delecciones o mutaciones predefinidas en determinados genes de especies ganaderas (por transferencia nuclear o por mutagénesis mediante el agente alquilante N-etil-N-nitrosourée, por ejemplo), lo que haría posible en un futuro la cría selectiva de animales resistentes a enfermedades infecciosas.

Palabras clave
References


