Microbial Considerations in Genetically Engineered Mouse Research

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Abstract

Microbial infections have long been of concern to scientists using laboratory rodents because of their potential to confound and invalidate research. With the explosion of genetically engineered mice (GEM), new concerns over the impact of microbial agents have emerged because these rodents in many cases are more susceptible to disease than their inbred or outbred counterparts. Moreover, interaction between microbe and host and the resulting manifestation of disease conceivably differ between GEM and their inbred and outbred counterparts. As a result, infections may alter the GEM phenotype and confound interpretation of results and conclusions about mutated gene function. In addition, because GEM are expensive to produce and maintain, contamination by pathogens or opportunists has severe economic consequences. This review addresses how microbial infections may influence phenotype, how immunomodulation of the host as the result of induced mutations may modify host susceptibility to microbial infections, how novel host:microbe interactions have led to the development of new animal models for disease, how phenotype changes have led to the discovery of new pathogens, and new challenges associated with prevention and control of microbial infections in GEM. Although the focus is on naturally occurring infections, extensive literature on the use of GEM in studies of microbial pathogenesis also exists, and the reader is referred to this literature if microbial infection is a suspected culprit in phenotype alteration.

Key Words: genetically engineered; immunomodulation; knockout; mice; microbial infections; pathology; phenotype; transgenic; virus

Introduction

Microbial infections have long been recognized for their potential to alter phenotypes. Infections that result in premature death or clinical signs of disease have obvious impact on phenotype and research using contaminated animals. In other cases, infections may be subclinical but alter the normal microscopic anatomy of infected animals. Subclinical infections also pose a significant risk to the research conducted with affected animals because they can alter the physiology (notably immune function) of experimental subjects or experimental endpoints such as tumor kinetics. These adverse effects have been summarized in a number of reviews (Baker 1998, 2003; Lipman and Perkins 2002; Nicklas et al. 1999; NRC 1991). With the advent of gene array tools, recognition of altered homeostasis has also extended to differential gene expression (Myles et al. 2003).

With the explosion of genetically engineered mice (GEM1), new concerns about the impact of microbial agents have emerged because these rodents may be more susceptible to infection than their inbred or outbred counterparts. Moreover, interactions between microbe and host and the resulting manifestations of disease may differ between GEM and their inbred or outbred counterparts. Alteration of phenotype may occur with microbes that are known to cause disease (pathogens) or with opportunists. In this article, the definition of opportunist proposed by Smith (1998) is used—an organism capable of causing disease only in a host whose resistance is lowered, for example by other diseases, drugs, or genetic manipulation. The latter is critical to a discussion of GEM inasmuch as these animals may have phenotypes associated with organisms previously unrecognized as opportunists.

Microbial Influence on Phenotype

Mice with spontaneous mutations that result in deficiencies in T cells (athymic nude [nu/nu] mice) and T and B cells (severe combined immunodeficiency [SCID1] mice) are notoriously susceptible to infections by the ubiquitous opportunistic fungus Pneumocystis carinii (Pneumocystis murina [Keely et al. 2004]), opportunistic bacteria such as the pas-
teurellas and staphylococci, and viral pathogens such as mouse hepatitis virus (MHV\textsuperscript{1}). Moreover, differential susceptibilities to infectious disease among inbred strains are commonplace and in many situations may be due to abnormal immune responses. For example, natural killer cell deficiencies in SJL mice, complement component 5 deficiencies in A, AKR, DBA/2, and SWR mice, and toll-like receptor 4 deficiencies in C3H/HeJ mice may render these mice susceptible to some infectious diseases (Barthold 2002; Poltorak et al. 1998). Differential susceptibility may also result from differences in host receptors utilized by pathogens. For example, SJL mice possess a different allelic form of the MHV receptor, rendering them resistant to infections by this virus compared with other strains of mice (Nedellec et al. 1994; Ohtsuka et al. 1996).

Based on the susceptibility of immunodeficient and inbred mice to certain infections, it is reasonable to anticipate that mice with induced mutations (i.e., knockout mice) in immune system effector molecules may also be more susceptible to the same infections, and when infected, their phenotype may be markedly altered. Examples abound, as do the types of pathogens involved in these novel host:microbe relationships. Many of the pathogens discussed in the following paragraphs and listed in Table 1 are indeed prevalent in contemporary colonies, including \textit{P. carinii}, \textit{Pasteurella pneumotropica}, \textit{Staphylococcus aureus}, MHV, minute virus of mice (MVM\textsuperscript{1}), \textit{Syphacia} spp., and \textit{Helicobacter} spp. (Jacoby and Lindsey 1998; Livingston and Riley 2003). It should not be assumed that less common agents not discussed (e.g., Sendai virus) do not have the potential to alter

| Table 1 Examples of naturally occurring infections associated with phenotype alterations in genetically engineered mice (GEM) |
|-----------------|-----------------|-----------------|-----------------|
| Agent           | Mutation\textsuperscript{a} | Phenotype                        | Reference (see text) |
| Bacterial infections |                  |                               |                  |
| \textit{Pasteurella pneumotropica} | Cd28             | Orbital abscesses               | Artwohl et al. 2000 |
|                 | \textit{lilrb} (CXCR2) |                              | Unpublished       |
|                 | E2F4             |                                | Humbert et al. 2000 |
| \textit{Staphylococcus aureus} | \textit{Plau} (uPa) | Furunculosis                   | Shapiro et al. 1997 |
|                 | LAT             |                                | JM Ward (personal communication) |
| Helicobacter hepaticus | \textit{ll10} (IL-10) | Typhlocolitis                  | Kulberg et al. 1998 |
|                 | \textit{Tcra} (TCR-\textalpha) |                         | Foltz et al. 1998 |
|                 | \textit{Tcbr} (TCR-\textbeta) |                           |                  |
|                 | \textit{TGft} (TGF-\textbeta)/Rag2 |                   |                  |
|                 | GPX             | Colitis and colon cancer       | Engle et al. 2002 |
| Viral infections |                  |                               | Chu et al. 2004   |
| \textit{Minute virus of Mice (MVM)} | \textit{Igh-6} (muMT, \textmu MT) | Death and anemia               | Naugler et al. 2001 |
| \textit{Mouse hepatitis virus (MHV)} | \textit{Ifng} (IFN-\gamma) | Granulomatous peritonitis      | France et al. 1999 |
|                 | \textit{Tnf} (TNF) | MHV persistence                | Pullium et al. 2003 |
|                 | \textit{Tcbr} (TCR-\textbeta) |                             | Rehg et al. 2001  |
| Murine norovirus (MNV) | \textit{Stat1} | Death                          | Karst et al. 2003 |
| Parasite infections | \textit{Stat6} | Altered host specificity       | GP Boivin (personal communication) |
| \textit{Syphacia muris} |                  |                               |                  |
| Fungal infections |                  |                               |                  |
| \textit{Pneumocystis carinii} | \textit{Cd40l} (CD40L) | Exudative alveolitis           | Furuta et al. 2001 |
| \textit{Trichosporon beigelii} | \textit{Ncf1} | Multiorgan pyogranulomatous inflammation | Lacy et al. 2003 |
| \textit{Paecilomyces variotii} | \textit{Cybb} | Pulmonary abscesses            | France and Muir 2000 |
| \textit{Zygomycetes fungi} | \textit{Irf1} (IRF-1) | Granulomatous gastritis        | Trottier et al. 2000 |
| Dual infections |                  |                               |                  |
| \textit{P. carinii} and \textit{P. pneumotropica} | \textit{Igh-6} (muMT, \textmu MT) | Exudative alveolitis/suppurrative bronchopneumonia | Marcotte et al. 1996 |
| \textit{H. hepaticus} and MHV | \textit{Igh-J} | Interstitial pneumonia         | Macy et al. 2000  |
|                 | \textit{Ifng} (IFN-\gamma) | Altered MHV disease severity   | Compton et al. 2003 |

\textsuperscript{a}uPA, urokinase-type plasminogen activator; LAT, linker of activation of T cells; TCR, T cell receptor; GPX, glutathione peroxidase; IFN-\gamma, interferon gamma; IRF, interferon regulatory factor; Igh, immunoglobulin heavy chain.
phenotype. Numerous studies have used experimentally infected GEM as tools in the study of the biology of murine pathogens, and these mice often exhibit increased susceptibility to infections and altered phenotypes as the result of these infections. Thus, the lack of reports on certain agents may reflect the low occurrence of these infections in contemporary colonies rather than their inability to alter phenotype.

Nine characteristics of microbial infections are listed in Table 2. In the text below, these characteristics are discussed with regard to the following: how microbial infections may influence phenotype, how immunomodulation of the host as the result of induced mutations may modify host susceptibility to microbial infections, how novel host-microbe interactions have led to the development of new animal models for disease, and how phenotype changes have led to the discovery of new pathogens.

**Characteristic 1: Opportunist infections of GEM may result in diseases similar to those seen in spontaneously mutated immunodeficient mice.**

*P. carinii* is a well-defined pathogen of T-cell-deficient, nude, and T- and B-cell-deficient SCID mice. Infections in these mice result in alveolitis characterized by the filling of alveoli by foamy exudate that contains abundant organisms and few host inflammatory cells (primarily macrophages) (Percy and Barthold 2001b). The life span of these mice is in turn shortened, and their utility in certain studies (i.e., those involving the respiratory tract) is severely compromised. *P. carinii* has also emerged as a problematic opportunist in GEM (Furuta et al. 2001; Macy et al. 2000; Marcotte et al. 1996). This development is likely due to its prevalence in colonies of immunocompetent mice and the fact that many GEM have predicted or unpredicted defects in immune responses. Recently, *P. carinii* exudative alveolitis, similar to that seen in SCID and nude mice, was identified in CD40 ligand (CD40L) knockout mice (Furuta et al. 2001). CD40L is expressed on T cells, and engagement with CD40 on antigen-presenting cells (APCs) results in activation of both T and B cells (Grewal et al. 1997). Experimental studies have demonstrated the critical role of both B and T cell activation as well as other cellular interactions (i.e., T cell: APC) via CD40:CD40L interactions in defense against *P. carinii* (Lund et al. 2003; Wiley and Harmsten 1995). Thus, it is not surprising that these CD40L knockout mice developed *P. carinii* pneumonia.

Like *P. carinii*, *P. pneumotropica* is an opportunist that may cause disease in immunodeficient mice. Infections of immunodeficient mice often manifest as conjunctivitis, otitis, or multiorgan abscess formation (Figure 1) (Percy and Barthold 2001a). Likewise, *P. pneumotropica* infections have resulted in disease in several mutant mice including CD28, CXCR2, and E2F4 knockouts (Artwohl et al. 2000; Humbert et al. 2000). CD28 is expressed on T cells, and engagement with its ligand, B7, on APCs results in T cell activation (Abass and Lichtman 2003a). Artwohl and colleagues (2000) described an outbreak of orbital abscesses in STOCK-Cd28tm1/nmack mice from which *P. pneumotropica* was isolated. These authors concluded that the lack of CD28 may have impaired normal T cell activation and rendered mice more susceptible to this infection.

Coworkers and I have seen a high incidence of deaths due to *P. pneumotropica* pneumonia in a colony of CXCR2 knockout mice (*IL8rb* gene mutation). Affected mice were homozygous for the mutation and of a mixed genetic background (CBA:Cg-Il8rb<sup>tm1Mwm</sup>/J); mice heterozygous for the mutation and wild-type counterparts were unaffected. Murine CXCR2 is the receptor for several CXC chemokines (e.g., KC and MIP-2) and is important in recruitment of neutrophils to sites of inflammation (Moore et al. 2000; Tsai et al. 2000; Zhang et al. 2001). Neutrophils are critical innate immune effector cells in the control of bacterial infections. The importance of CXCR2 in infectious disease was demonstrated in mice with experimental Lyme arthritis wherein neutrophils were marginalized within blood vessels of joints but did not enter tissues (Brown et al. 2003). Moreover blockade of CXCR2 in several murine models of bacterial pneumonia resulted in significant increases in mouse mortality associated with marked decreases in neutrophils recruitment (Moore et al. 2000; Tateda et al. 2001; Tsai et

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**Table 2 Characteristics of microbial infections of genetically engineered mice (GEM)**

1. Opportunist infections of GEM may result in diseases similar to those seen in spontaneously mutated immunodeficient mice.
2. Some infections of GEM may be caused by common opportunists.
3. Pathogens known to cause disease in immunocompetent mice are likely to cause disease in many GEM; however, in some cases the lesion manifestation is unusual.
4. Genetic manipulation may lead to heightened susceptibility to clinical disease in infections with agents that normally result in asymptomatic infections.
5. Genetic manipulation of the host may affect host specificity of certain pathogens.
6. Combined infections may result in unusual or new GEM phenotypes.
7. Microbe-associated phenotypes are not necessarily due to immune defects.
8. Unrecognized infections may complicate interpretation of results from experiments that use contaminated rodents.
9. Differences in microbial flora or genetic background may have an impact on GEM phenotypes.
al. 2000). Thus, it is likely that the CXCR2 knockout mice with *P. pneumotropica* pneumonia were unable to mount an appropriate and timely neutrophil-mediated defense against this opportunistic.

An outbreak of *P. pneumotropica* conjunctivitis was also reported in mice transgenic for carcinoembryonic antigen cell adhesion molecule (CEACAM1-1). *Ceacam1* is the gene encoding a signaling receptor that is implicated in several physiological functions, including immune functions (Markel et al. 2004; Schmitter et al. 2004; Skubitz et al. 2001). In addition, *Ceacam1* serves as a receptor for a number of bacterial and viral pathogens (Hill et al. 2005; Zeluz et al. 2003). Although it is tempting to speculate that the overexpression of this gene resulted in increased susceptibility to pasturellosis, the speculation is difficult to confirm because wild-type mice from the report cited above also developed disease (Matsumiya and Lavoie 2003). This uncertainty highlights that care should be taken when establishing the role of an infectious agent in any phenotype alteration, including those seen in GEM. When a microbial agent is found to be associated with phenotype alteration, it is tempting to implicate that agent in that phenotype change immediately. However, as described below, controlled studies are warranted to fully explore the role of these agents in phenotype alterations.

*S. aureus* represents another common colonizer of mice that may cause clinical disease in immunodeficient mice. Notably, nude mice can develop folliculitis and furunculosis of the muzzle leading to coalescing abscess formation often referred to as botryomycosis (Percy and Barthold 2001a). Likewise, certain strains of GEM have been found to have an increased incidence of staphylococcal botryomycosis. As an example, mice deficient in urokinase-type plasminogen activator (uPA) have been reported to develop staphylococcal folliculitis (Shapiro et al. 1997). uPA catalyzes the conversion of plasminogen to plasmin, which functions as a broad-spectrum serine protease with many functions, including inflammation modulation. The specific role of uPA in the response (immune and non-immune mediated) to bacterial infection has not been defined. The authors speculated that deficiency in uPA may have resulted in alterations of the local microenvironment of the skin, possibly through a general reduction in the activation or availability of cytokine growth factors. This altered microenvironment in turn facilitated bacterial invasion and infection. Botryomycotic lesions have also been seen in mice deficient in linker of activation of T cells (LAT) (J. M. Ward, Center for Cancer Research, National Cancer Institute, Frederick, MD, September 2003). Given that LAT is important in T cell activation (Abass and Lichtman 2003a) and that T cell-deficient nude mice are susceptible to botryomycosis, it is not surprising that mice deficient in LAT would develop a similar condition. Moreover, the identification of botryomycosis in both LAT-deficient and nude mice further strengthens the supposition that T cells are critical in defense against staphylococcal-induced diseases.

The number of reports of opportunistic infections by other common bacterial species is relatively low. When one considers that mice and rats harbor a number of purported opportunists (e.g., bacterial species of the genera *Klebsiella*, *Pseudomonas*, and *Proteus*) and a number of protozoal species (e.g., those of the genera *Giardia*, *Trichomonas*, and *Entamoeba*), it is curious that to date, no adverse affects of these colonizers have been documented in GEM. This lack may reflect a lack of reporting of such complications or highlight that these agents are best regarded as commensals and only when they breech physical immune barriers do they have the potential to cause disease.

**Characteristic 2: Some infections of GEM may be caused by less common opportunists.**

The examples described above represent complications associated with well-defined and relatively common rodent opportunistic pathogens. Infections by uncommon opportunists have also occurred in GEM. For example, pulmonary abscesses due to the common environmental fungus *Paecilomyces variotii* were identified in a colony of cytochrome b-245, beta polypeptide (*Cybb* or *gp91phox*−/−) mice, which lack functional NADPH oxidase, the enzyme complex responsible for generating the respiratory burst in phagocytes (France and Muir 2000). In another report, a survey of B6.129S6-Cybbtm1Din mice revealed that they were susceptible to a number of fungal as well as bacterial opportunistic infections (Bingel 2002). Multiorgan pyogranulomatous inflammation due to the saprophytic fungus *Trichosporon beigelii* was also identified in C57BL/6Nai mice with targeted mutations in neutrophil cytotoxic factor 1 (*Ncf1* or *p47phox*−/−), a gene encoding a second critical component of the NADPH oxidase complex (Lacy et al. 2003). These cases highlight and reinforce the importance of innate immunity in fungal defense.

Uncommon opportunists may also alter the phenotype of mice with targeted mutations in genes encoding molecules involved in differentiation of T helper (Th1) cell...
subsets. For example, interferon regulatory factor (IRF\(^1\))-1 knockout mice were found to have severe granulomatous gastritis (Figure 2, A and B). Examination of tissue sections with silver stains revealed fungal hyphae morphologically consistent with fungi of the class Zygomycetes (Trottier et al. 2000). IRF-1 is a transcriptional activator for interferon systems, and IRF-1 knockouts have reduced T cell receptor (TCR\(^1\))αβ+, CD4-, CD8+ T cells and decreased interferon gamma (IFN-\(\gamma\))-induced levels of macrophage-inducible nitric oxide synthase mRNA. As a result, these mice are deficient in their ability to mount normal Th1 immune responses and they typically mount reciprocal Th2 responses (Lohoff et al. 1997; Taki et al. 1997). Because the normal immune response to fungal infections is a Th1 response, it is likely that this mouse was unable to mount the appropriate Th1 defense against this environmental fungus and instead mounted an ineffective Th2 response. This case exemplifies that targeted mutations in genes encoding effector molecules of specific arms of the immune responses may result in susceptibilities to uncommon opportunists.

Curiously, many of the fungal pathogens described above have not been reported to cause disease in the more commonly used spontaneously immunodeficient mice. This lack likely reflects the complex interplay between innate immunity, which SCID and nude mice possess, and adaptive immunity in protection against fungal agents. These reports of opportunistic fungal infections in GEM highlight the importance of expanding the list of potential opportunistic infections in GEM well beyond those previously described in spontaneously immunodeficient mice.

Characteristics 3: Pathogens known to cause disease in immunocompetent mice are likely to cause disease in many GEM; however, in some cases the lesion manifestation is unusual.

MHV represents numerous genetically related strains of viruses that vary in their virulence and organ tropism. MHV infection usually results in transient and subclinical infections in immunocompetent hosts. In immunodeficient mice, wasting syndromes with characteristic hepatic or intestinal lesions have been described (Percy and Barthold 2001c). Recently, a previously uncommon presentation of MHV infection was reported in IFN-\(\gamma\) knockout mice. These mice developed depression, variable ascites, and mild diarrhea (France et al. 1999). Necropsy examinations revealed granulomatous peritonitis and pleuritis with extensive adhesion formation. Curiously, the parenchymal lesions and syncytia formation commonly seen in MHV infections were uncommon in infected IFN-\(\gamma\) knockout mice. IFN-\(\gamma\) exhibits antiviral properties and activates innate immunity and the subsequent development of effector CD8+ T cells; all of these functions are critical to host defense against viral pathogens (Abass and Lichtman 2003b). Thus, it is logical that deficiency in IFN-\(\gamma\) would render mice more susceptible to viral infections such as MHV. However, why a deficiency in IFN-\(\gamma\) resulted in an unusual presentation remains unknown. Importantly, investigations of the mechanisms of this unusual presentation may provide new insight to pathogenesis of this disease as well as those of other

Figure 2 (A) Interferon regulatory factor (IRF)-1 knockout mouse with severe granulomatous gastritis (arrow). (B) Photomicrograph of stomach wall demonstrating granuloma with intralesional giant cells (inset), and (C) fungal hyphae morphologically consistent with fungi of the class Zygomycetes.
coronaviral infections, notably feline infectious peritonitis virus infection, which often manifests as peritonitis. This report highlights that diagnostically challenging uncommon presentations of infectious disease can occur in GEM.

**Characteristic 4: Genetic manipulation may lead to heightened susceptibility to clinical disease in infections with agents that normally result in asymptomatic infections.**

Characteristic 4 is best exemplified by the manifestation of murine parvovirus infections in NOD.Cg-\textsuperscript{H2\textsuperscript{H4}}-Igh-6\textsuperscript{−/−} mice. These mice have defects in B cell maturation due to the targeted mutation of the immunoglobulin heavy chain-6 (Igh-6\textsuperscript{1}) gene. In this case, several 3- to 10-wk-old NOD.Cg-\textsuperscript{H2\textsuperscript{H4}}-Igh-6\textsuperscript{−/−} mice presented with lethargy, hunched posture, and runting and died (Figure 3A) (Naugler et al. 2001). Wild-type mice and mice heterozygous for the Igh-6 mutation as well as several strains with mutations in other immune effector molecules were unaffected. These colonies historically had endemic MVM and mouse parvovirus (MPV\textsuperscript{1}) infections. Histological examination of tissues from affected knockout mice revealed large intranuclear inclusions in mononuclear cells of the bone marrow and spleen (Figure 3B). Using agent-specific polymerase chain reaction (PCR\textsuperscript{1}) assays, mesenteric lymph nodes and spleens were found to be positive for MVM in all animals and MPV in some animals. Ultrastructural and immunohistochemical examinations demonstrated that inclusions contained 20-nm virions that were positive for the nonstructural parvoviral protein NS-1 and the viral protein 2 capsid antigen of MVM (Figure 3B). The clinical disease in affected mice was subsequently linked to anemia and leukopenia, suggesting that the MVM (possibly in conjunction with MPV) had infected hematopoietic progenitor cells in the spleen and bone marrow. In the natural setting, MVM infections are almost universally subclinical, although experimental infections of SCID mice and neonatal mice may result in myelosuppression similar to that seen in the Igh-6 null mice (Lamana et al. 2001; Segovia et al. 1995, 1999, 2003). The finding that NOD.Cg-\textsuperscript{H2\textsuperscript{H4}}-Igh-6\textsuperscript{−/−} mice were more susceptible to clinical disease when infected with MVM suggests that B cell immunity is important in limiting disease due to this agent. However, determination of the exact role of B cells in MVM immunity awaits further investigation.

**Characteristic 5: Genetic manipulation of the host may affect host specificity of certain pathogens.**

The examples above demonstrate increased sensitivity to or unusual manifestations of disease when known rodent pathogens or opportunists infect GEM. Genetic mutation may also alter host specificity of certain infection agents. As an example, Boivin (G.P. Boivin, University of Cincinnati, Cincinnati, OH, personal communication, September 2003) observed novel helminth infections in C57BL/6;129 mice with targeted mutations in signal transducer and activator of transcription 6 (Stat6\textsuperscript{1}). Mice were found with worms protruding from their anuses and subsequently found to be heavily colonized by pinworms. However, the pinworms were not *Syphacia obvelata*, the mouse pinworm, but rather were identified as *Syphacia muris*, the rat pinworm. Examination of other colonies in the facility revealed that rats in two adjacent animal rooms were infected with *S. muris*, while no other mice in the facility were infected, including knockout mice lacking other immune effector molecules. Stat-6 mediates signal pathways of interleukin (IL\textsuperscript{1})\textsuperscript{3}, IL-4, and IL-13, Th2 cytokines that are important in antiparasite defense. Thus, it is likely that this Th2 response was altered.
in Stat-6 null mice and that they were rendered susceptible to helminth parasites that are primarily specific to another genus of rodents. Curiously, mice with targeted mutations in genes encoding other Th2 effector molecules, including IL-4 receptor alpha (IL4ra), did not become infected with S. muris, highlighting the complexity of this response and the unpredictability of these novel host: pathogen interactions.

**Characteristic 6: Combined infections may result in unusual or new GEM phenotypes.**

Several reports have described novel disease manifestations in GEM infected with two or more murine pathogens (Compton et al. 2003; Macy et al. 2000; Maggio-Price et al. 1998; Marcotte et al. 1996). Marcotte and colleagues (1996) and Macy and coworkers (2000) described combined *P. pneumotropica* and *P. carinii* infections in B cell-deficient GEM. In the first report, mice with mutations in the µMT (*Igh-J*) gene developed exudative alveolitis typical of *P. carinii* infection and in some cases, *P. pneumotropica*-induced suppurative bronchopneumonia. In contrast, Macy and coworkers described diffuse interstitial pneumonia with minimal evidence of the eosinophilic exudates that typifies *P. carinii* infections in mice deficient in the gene for the heavy chain joining region *Igh-J*. Moribund mice in the latter case also exhibited pyogranulomatous pneumonia. These cases highlight the spectrum of possible disease manifestations that may be seen in mice with very similar immunodeficiencies.

In another report, IFN-γ knockout mice co-infected with MHV-G and *Helicobacter hepaticus* developed pleuritis, peritonitis, hepatitis, pneumonia, and meningitis (Compton et al. 2003). To ascertain whether co-infection by *H. hepaticus* modulated MHV-G-induced disease, investigators performed experimental studies. Curiously, *H. hepaticus* infection appeared to reduce the severity of MHV-G-induced lesions during acute infection, but exacerbated some lesions (hepatic and meningitis) at more chronic stages of disease. The authors speculated that because of their common intestinal niche, *H. hepaticus* may have altered replication kinetics of MHV-G or altered local immune cell infiltration or cytokine environment such that MHV-G-induced disease severity was modulated.

**Characteristic 7: Microbe-associated phenotypes are not necessarily due to immune defects.**

*P. pneumotropica* was also implicated in neonatal rhinitis in a colony of mice with targeted mutations in the E2F transcription factor E2F4 (Humbert et al. 2000). However, rather than a defect in the immune system, the authors speculated that the increased susceptibility of these mice to bacterial infections was due to intrinsic defects in craniofacial bone abnormalities that lead to sequestering of fluid in the nasal cavity and associated increase in nasal cavity pressure.

**Characteristic 8: Unrecognized infections may complicate interpretation of results from experiments that use contaminated rodents.**

Characteristic 8 is best exemplified when one reviews the history of rodent helicobacters. *H. hepaticus* was first recognized as a pathogen because it had complicated an ongoing research project. An increased incidence of hepatic adenomas and previously unrecognized chronic active hepatitis were noted in untreated control mice on a large long-term study to evaluate the carcinogenic potential of a chemical compound. Examination of silver-stained sections of liver revealed slender curved to spiral bacteria associated with lesions. A bacterium was subsequently cultivated from the intestinal tract and liver, characterized as a previously unrecognized member of the genus *Helicobacter*, and named *H. hepaticus* (Fox et al. 1994). Because the carcinogenic potential of chemical compounds is evaluated by the induction of hepatic tumors, this dramatic increase in hepatic neoplasia associated with *H. hepaticus* infection invalidated the results of this and other long-term carcinogenesis studies (Hailey et al. 1998; Ward et al. 1994).

Subsequently it was shown that *H. hepaticus* infections also complicated research of the intestinal tract. Infected immunodeficient SCID and nude mice developed a high incidence of rectal prolapse and chronic proliferative typhlocolitis and proctitis, in addition to the aforementioned hepatitis (Li et al. 1996; Russell et al. 1995; Ward et al. 1996). Additional studies revealed that some immunocompetent strains of mice may also develop intestinal disease (Fox et al. 1996; Livingston et al. 2004; Myles et al. 2003; Whary et al. 1998) or gallstones (Maurer et al. 2005) when infected with this bacterium. Moreover, both enteric and hepatic diseases have been associated with alterations in gene expression, even when no clinical or histological disease is evident (Myles et al. 2003). Thus infection with this bacterium has far-reaching implications in research involving these organ systems.

Given this history, it is easy to see in hindsight that *H. hepaticus* had great potential to modify phenotypes of GEM and to complicate interpretation of research results from infected rodents. Concurrent with the recognition of *H. hepaticus* as a pathogen of rodents, several genetically engineered strains of mice were being created and many were found to have chronic inflammation of the intestinal tract. Subsequently, many—including those with targeted mutations in genes encoding IL-10, TCR-α, β2 microglobulin, intercellular adhesion molecules, transporter associated with antigen processing (MHC1 deficient), and uPA, as well as those heterozygous for mutations in p53—were found to be colonized by *H. hepaticus* (Foltz et al. 1998). In some cases, inflammation of the intestinal tract abated or was...
eliminated when these strains were rendered helicobacter free (Foltz et al. 1998; Kullberg et al. 1998). Moreover, experimental infection of IL-10 and TCR-αβ knockout mice with *H. hepaticus* caused more severe disease, further supporting the role of this bacterium in induction of intestinal inflammation and its potential to confound interpretation of results from inflammatory bowel disease research (Burich et al. 2001; Chin et al. 2000; Kullberg et al. 1998).

*H. hepaticus* infections also complicated the cancer phenotype of mice with mutations in both transforming growth factor β1 (*Tgfb1*) and recombaine activating gene 2 (*Rag2*). These mice developed colonic inflammation and cancer; however, when rendered germ free, these lesions did not develop (Engle et al. 2002). To discern the role of *H. hepaticus* in inflammation and cancer, germ-free *Tgfb1−/−Rag2−/−* mice were reintroduced into either *H. hepaticus*-containing environments or *H. hepaticus*-free environments. Only when introduced into the *H. hepaticus*-containing environment did lesions reappear, suggesting that this bacterium was a key trigger to intestinal inflammation and subsequent cancer. Similarly, the incidence and location of inflammation-associated intestinal cancer differed between germ-free mice deficient in the glutathione peroxidase isoymes Gpx-1 and Gpx-2 that were either reconstituted with commensal flora or raised or weaned into “non-specific pathogen free” conditions that included *Helicobacter* spp. contamination (Chu et al. 2004).

The recognition of *H. hepaticus*-associated diseases led to the discovery of several other rodent helicobacters, many of which have also been associated with disease in GEM (Burich et al. 2001; Fox et al. 1999; Maggio-Price et al. 2002). In addition, the recognition of *H. hepaticus* as an organism capable of altering the phenotype of GEM has led investigators to question whether research using other genetically altered species is complicated by similar infections. For example, elimination of *Helicobacter* spp. from a colony of diabetes-prone rats led to elimination of the clinical, but not histological, diabetes in these rats, leading the authors to speculate that this bacterium may have altered the phenotype of this model (Baran et al. 2004).

Characteristics 9: Differences in microbial flora or genetic background may have an impact on GEM phenotypes.

Some controversy exists about the role of *H. hepaticus* in intestinal inflammation. Although many studies have shown that *H. hepaticus* can initiate intestinal inflammation in a number of GEM, Dieleman and colleagues (2000) found no differences in severity of intestinal inflammation between *H. hepaticus*-infected and helicobacter-free IL-10-deficient mice. This result was in sharp contrast to the findings of Kullberg and coworkers, who found that *H. hepaticus* infection exacerbated intestinal inflammation in IL-10-deficient mice (Kullberg et al. 1998). Although there are many plausible explanations for this discrepancy, one possibility is that the flora of these mice differed in comparison with mice used in previous studies. This possibility adds a whole new potential variable to studies of intestinal inflammation that use GEM. Indeed, it is well established that intestinal inflammation of many models is abrogated when these mice are rendered germ free and in some cases specific pathogen free (Chu et al. 2004; Engle et al. 2002; Kado et al. 2001; Schultz et al. 1999; Sellon et al. 1998). Moreover, other animal models of chronic inflammation, including experimental allergic encephalomyelitis, adjuvant arthritis, and collagen-induced arthritis, are also modified when animals are rendered germ free, suggesting that intestinal flora modulate systemic immunity in addition to mucosal-associated immune responses (Birnbaum et al. 1998; Bjork et al. 1994; Breban et al. 1993; Kohashi et al. 1979, 1986a,b; Taurog et al. 1999; van de Langerijt et al. 1993). Collectively, these observations have prompted some investigators to propose that gnotobiotic (known flora) animals should be used in studies of inflammatory conditions that use GEM. However, the cost of producing and housing these animals, especially when coupled with inherent costs of GEM production and maintenance, often precludes their use. At the very least, the possible role of intestinal flora should be considered in situations where inflammatory phenotypes change unexpectedly or differ among laboratories.

It is well established that genetic background can influence the phenotype of GEM (Barthold 2004; Cianflone et al. 2003; Huang et al. 1999; Kahn et al. 2000; Lahvis and Bradfield 1998; Linder 2001). Thus, it is not surprising that genetic background may also influence microbial-associated phenotypes. This effect was recently exemplified in studies of helicobacter-associated intestinal cancer. In these studies, 129/SvEi Rag2-deficient mice developed more severe *H. hepaticus*-induced colitis and epithelial dysplasia than Rag-2-deficient BALB/c or C57BL/6 mice (Erdman et al. 2003a). Others have also speculated that background strain may have influenced the type and/or degree of phenotype alterations seen in GEM when infected with pathogens. As described above, Marcotte and colleagues (1996) and Macy and coworkers (2000) described combined *P. pneumotropica* and *P. carinii* infections in very similar B cell-deficient GEMs (*Igh-6* and *Igh-J* mutations). However, the disease phenotype exhibited by these mutants differed markedly: The *Igh-6* null mutants developed exudative alveolitis and suppurative bronchopneumonia, and the *Igh-J* null mutants developed primarily interstitial pneumonia with minimal evidence of the exudate. Macy and coworkers (2002) speculated that the differing genetic backgrounds of these two mutants (C.B-17 and MRL-*Fas* for the *Igh-6* null mutant, and C57BL/6 for the *Igh-J* null mutant) may have contributed to the differences in disease phenotype.

A “domino effect” of infectious disease on phenotype that demonstrates the role of genetic background has also been proposed (Barthold 2002). To this end, arylhydrocarbon (*Ahr*) knockout mice on a C57BL/6 mouse background develop an immunodeficiency associated with
defective DNA repair that results in chronic skin, gastrointestinal, and genitourinary infections. These mice also develop unexplained cardiomyopathy and congestive heart failure (Fernandez-Salguero et al. 1997). Barthold (2002) proposed that the immunodeficiency in these mice led to opportunistic infections that accelerated the onset of multisystemic amyloidosis, normally a late-onset disease in C57BL/6 mice. Renal amyloidosis in turn may have predisposed these mice to coagulopathies and subsequent atrial thrombosis, cardiomyopathy, and heart failure. This scenario could also occur in other common background strains that are prone to amyloidosis (i.e., FVB) or atrial thrombosis (i.e., BALB/c) and highlights the complexity with which microbial infections may complicate phenotypes (Barthold 2002).

Other Emerging Infections

The profound impact of *H. hepaticus* infections on GEM phenotypes and research using GEM was not immediately recognized due to several factors. The bacterium was discovered concurrently with the increasing use of genetically engineered rodents. It was subsequently found to be prevalent in rodent colonies and this finding, coupled with the popular practice of “sharing” GEM, likely led to exposure of many different strains. Because *H. hepaticus* colonizes most rodents without causing clinical disease, infections were not readily apparent. Moreover, histological disease only developed with chronic infection. Finally, some strains of mice, notably C57BL/6, are resistant to histological disease. Thus, complications due to *H. hepaticus* infection were not readily apparent until research devoted to assessing its role in disease phenotype was initiated. The *H. hepaticus* example highlights the potential for emerging pathogens to remain insidious and complicate interpretation of phenotypes in GEM. Recurrence of this scenario with other emerging pathogens/opportunists is not only possible but is also likely. Thus, the rodent diagnostician must be vigilant of phenotype alterations that conceivably are complicated by contamination with as yet undefined infectious agents.

Novel Host:Pathogen Interactions in GEM Can Lead to Development of New Animal Models

While the examples described above highlight how pathogens or opportunists may complicate interpretation of results obtained from GEM, they also exemplify how discovery of such novel host:pathogen interactions may be exploited in the development of new animal models. *H. hepaticus* has emerged as a model bacterial organism capable of initiating chronic Th1-mediated inflammation in the intestinal tract and liver. Moreover, through experimental infections, interactions between *Helicobacter* spp. and GEM strains, including those deficient in Mdr1a, Rag-2 (on a 129/SVEv background), IL-7, NF-kb (p50−/−p65+/−), and TCR-α, led to the development of new animal models for inflammatory bowel disease (Burich et al. 2001; Chin et al. 2000; Erdman et al. 2001; Kullberg et al. 1998, 2001, 2002, 2003; Maggio-Price et al. 2002; Tomczak et al. 2003; von Freedeen-Jeffry et al. 1998) and chronic inflammation-associated intestinal dysplasia/cancer (Erdman et al. 2003a,b). In summary, naturally occurring infections of GEM should always be viewed with the “glass half full” eye. Many of these infections have great potential to be exploited as novel animal models of disease.

Altered GEM Phenotypes Can Lead to Discovery of Novel Infectious Agents

The recognition of increased deaths in a colony of mice deficient in Stat1 and Rag2 led to the discovery of murine norovirus (MNV-1) (Karst et al. 2003). The virus was identified by representational difference analysis (RDA) (Lisitsyn 1995; Pastorian et al. 2000) and was ultimately isolated and purified. Experimental inoculations with purified MNV-1 resulted in deaths in mice deficient in Stat1, Stat1 and protein kinase RNA activated, Stat1 and Rag2, and αβ and γ receptors of IFN (IFNαβγR). Mice with mutations in a number of other innate immune effector molecules survived infection. Stat1 is involved in signaling through both IFN αβ and IFNγ receptors, so collectively these findings implicate Stat1-dependent innate immunity in prevention of lethal MNV-1 infection. This case highlights that unexplained/unexpected disease, death, or changes in phenotype may indicate infections by previously unrecognized microbial agents. The scenario also highlights how aggressive pursuit of novel agents with contemporary molecular tools such as RDA is warranted in situations where phenotypes of GEM have changed. Moreover, this discovery has provided a new and exciting animal model for the study of norovirus biology and disease pathogenesis.

Potential for Other Less Common Microbial Organisms to Affect Phenotype

The literature is replete with examples in which GEM are used in studies to investigate pathogenesis of infectious disease and conversely where well-characterized rodent pathogens are used as tools in conjunction with GEM to increase knowledge of basic animal biology. These studies indicate that many GEM are susceptible or develop novel manifestations of disease when infected with a number of rodent pathogens in addition to the prevalent agents described above. For example, infection of osteopontin and IFN-γ knockout mice with murine rotavirus demonstrated that rodents are highly susceptible to clinical disease when these genes are mutated (Rollo et al. 2005; Vancott et al. 2003). Moreover, infection of B-cell-deficient muMT (Igh-6) null mice with the once common pathogen *Mycoplasma pulmo-
nism results in arthritis or periarticular changes that resemble disease seen in human X-linked agammaglobulinemia (Berglof et al. 1997). Does the lack of reports of naturally occurring rotavirus or M. pulmonis-induced diseases in GEM reflect the low prevalence of these agents or experimental phenomena that are not recapitulated in the natural setting? It is likely that both factors (and others) may be involved, but these examples highlight that microbial agents have much greater potential to affect GEM phenotypes than has been recognized to date.

Implicating Infectious Agents in Phenotype Alteration

Implicating infectious agents as the cause of phenotype change requires fulfilling Koch’s postulates: identifying the suspect agent, associating colonization with phenotype alteration, and reproducing the phenotype alteration in experimental studies. To identify infectious disease in GEM, established tools used to identify infectious agents in any rodent may be utilized. Ideally, the agent should be cultured so that it can be used in experimental inoculation studies. However, for initial screening for infectious agents, other less labor-intensive and less expensive methods such as serology or PCR-based detection of microbial DNA may be used. As discussed below, negative results from serological analyses should be interpreted with caution because many GEM have defects in the immune response that render them incapable of producing a normal serological response or they may be on a genetic background that inefficiently se-roconverts to some pathogens (Besselsen et al. 2000; Compton and Riley 2001; Livingston and Riley 2003).

Identification of microbial pathogens in a GEM does not indicate that infection has altered the phenotype of that mouse. To do so requires correlation of infection with alteration of phenotype and controlled experimental studies. The agent should be isolated, and the GEM should be cleared of infection. Once cleared, animals should be examined for return to normal phenotype. Finally, groups of animals can be reinfect ed and compared with uninfected animals using appropriate phenotypic analyses (mortality rates, histological screening for lesions, gene expression analyses, experiment-specific endpoints, etc.).

Control of Infections in Colonies of GEM

The examples described above highlight some of the challenges faced in diagnosing infectious disease and in determining the role of microbes in alteration of phenotype in GEM. The development of sensitivities to opportunists or to novel disease presentations associated with known pathogens also highlights the critical importance of maintaining genetically engineered animals in pathogen-free conditions. These examples focus on clinical and/or histological disease. It should be noted that subclinical infections also have great potential to alter results obtained from infected genetically engineered animals. For example, gene dysregulation caused by infection may occur in the absence of clinical and histological disease (Myles et al. 2003). Because gene dysregulation is a key component of mechanistic research, the potential for infectious disease to alter this component has far-reaching implications. As a result, with the discovery of any phenotype perturbation in a colony of GEM, infections should always be considered as a potential cause of such perturbations and should be investigated appropriately.

Prevention of Infections in Colonies of GEM

To prevent introduction of infectious agents into colonies of genetically engineered mouse colonies, health monitoring and quarantine programs are of paramount importance. The components of traditional health monitoring programs include the assessment of incoming animals through vendor screening and/or quarantine testing, periodic routine assessment of resident animals via random screening or testing of dedicated sentinel animals, and the assessment of biological materials used in mouse experiments. A program for individual animal monitoring should also be in place to identify index cases of diseases or unexpected experimental results in the early stages of outbreaks. There is great variety in health surveillance programs; no two designs are identical, and the program design should cater to the needs of the institution. With the emergence of genetically engineered animals and the associated frequent transfer of these mice among institutions, consideration should also be given to the needs of other institutions that may receive mice from the home institution.

Caveats of Health Monitoring in GEM

Monitoring for introduction of infectious agents into colonies of GEM generally follows standard guidelines. However, the explosion of genetically engineered animals has directly or indirectly led to many new challenges in prevention of disease introduction. For example, the increased transfer of GEM among facilities and institutions, the associated increase in housing densities, and the housing of multiple “small colonies” have led to greater potential exposure to infectious agents. Increased susceptibilities to disease or persistence of disease may also serve to increase exposure of other mice concurrently housed with contaminated mice. Other challenges associated with prevention of disease introduction include the following: (1) the uncertain immune status of most GEM; (2) the inherent value and expense of replacing GEM; and (3) the use of new caging designs (e.g., ventilated cage systems) that minimize cage to cage transmission of infectious disease.

Sampling of colony or quarantine GEM for antibody production is not ideal because many of these strains have defects in the immune response that render them incapable
of producing a normal serological response. Because the immune system of many GEM is often not adequately characterized, it is prudent to consider all GEM to have potential immunodeficiencies (i.e., “immunovague”). Moreover, many genetic mutations are present on a C57BL/6 genetic background, and mice of this background do not adequately respond to some infections, notably MPV (Besselsen et al. 2000). Finally, the expense of GEM usually precludes their use as colony sentinels. Collectively these features of GEM suggest that their use in health monitoring is not warranted.

As an alternative, most GEM health monitoring utilizes sentinel-based programs. As with any sentinel program, negative results from these mice also must be interpreted with caution because soiled bedding transfer may not reliably transmit all agents (Artwohl et al. 1994; Cundiff et al. 1995; Dillehay et al. 1990), including H. hepaticus in some settings (Compton et al. 2004b). Contact sentinels offer one option; however, this practice can be very expensive. Moreover, sentinel mice can serve as a source of opportunists to GEM, especially those with immunodeficiencies. Thus, careful selection of pathogen- AND opportunist-free sentinels is prudent in the design of GEM health monitoring programs. In exceptional cases with colonies of very rare GEM, the use of known immunodeficient sentinels may be warranted to ensure protection of the colony.

Infectious agents may also persist longer in GEM, which may be advantageous to sentinel programs because the chances of infecting sentinels increases with longer persistence within the colony that is being monitored. This persistence is best exemplified with MHV infections. In immunocompetent mice, MHV is normally a self-limiting disease that is cleared within 1 to 2 mo (Barthold and Smith 1990). In contrast, immunodeficient mice may develop persistent chronic infections (Percy and Barthold 2001c). Likewise, two recent reports highlight that MHV may persist in some genetically engineered strains, including B6.129S1-Tnf^{−/−}\(l^{−}\) (tumor necrosis factor-deficient) and transgenic strains expressing a rearranged T-cell receptor β chain (Pulliam et al. 2003; Rehg et al. 2001). Importantly and unlike immunodeficient SCID and nude mice, these mice did not show clinical signs. In both cases, infections were successfully detected using sentinel monitoring programs. Thus, the use of well-designed sentinel-based health monitoring programs in GEM colonies offers many advantages over colony monitoring-based programs, and this practice has become commonplace in many GEM facilities.

The GEM-related issues described above (e.g., increased exposure to pathogens/opportunists and increased potential persistence of microbial colonization) are further complicated by the common use of ventilated caging systems. Because these systems isolate individual cages, they may minimize spread of infections (the “pro”) but also result in inefficient detection of infections by sentinel monitoring programs (the “con”). For example, in experimental studies assessing monitoring programs for ventilated systems, one strain of MPV was ineffectively transmitted to soiled bedding sentinels, in part due to a dilutional effect (Compton et al. 2004b). This dilutional effect could readily occur in a natural setting (i.e., via accidental introduction of a single GEM that is persistently colonized by a microbial pathogen) and result in ineffective detection of infections. Fortunately, new tools are being developed to monitor these systems, including the use of exhaust air sentinels, screening of filtered exhaust air using molecular diagnostic tools, and combinations of these methods with more traditional sentinel-based methods. The reader is referred to several publications for additional details (Compton et al. 2004a,b; Lipman and Homberger 2003).

Managing GEM Colony Disease Outbreaks

Rapid detection and prompt containment of contaminated colonies are critical to proper management of animal facilities that import and house GEM. Managing disease outbreaks in colonies of GEM also follows standard procedures and presents clinicians with new challenges. When sentinel testing or other evidence suggests infection by a particular agent, it is important to confirm that the agent is truly present in the colony (i.e., by testing additional sentinels or colony mice). Once an infection is verified, the room should be quarantined to prevent further spread to other rooms in the facility. Incoming shipments should be diverted to other areas, and transfers out of the room should be cancelled. A plan of action should also be developed by the veterinary and animal facility management groups in concert with others that are affected, such as the research groups holding animals in the area and use of common procedural rooms.

A variety of methods can be used to eliminate an infectious agent from a colony, and careful professional judgment is needed to determine the most appropriate course of action. Treatment of bacterial or fungal infections is often not warranted because most bacteria/fungi are difficult to eradicate completely with antibiotic/antifungal therapy, especially when mice are immunocompromised. Depopulation strategies that are often used in colonies of readily available inbred or outbred animals are often not suitable for colonies of GEM inasmuch as these mice may be difficult to replace. “Burnout” or cessation of breeding should be used with caution because these strategies are predicated on the lack of persistence of the agent. As mentioned above, some agents (notably MHV) may persist in GEM. Moreover, burnout strategies often employ serology to identify colonized animals accurately. Because the capacity of GEM to seroconvert to infectious agents is in most cases unknown, results of serological testing may be difficult if not impossible to interpret (see Caveats of Health Monitoring in GEM).

Because of the caveats described above, infectious disease outbreaks in colonies of GEM are most commonly managed by rederiving the strain using cesarean section or embryo transfer (Morrell 1999; Suzuki et al. 1996; Van Keuren and Saunders 2004). These rederivation strategies.
can be complemented by the use of antibiotics to minimize clinical signs of infections disease and allow for the production of breeding animals. For example, several investigators have used enrofloxacin treatment to decrease clinical signs of bacterial or P. carinii infections in preparation for rederivation of GEM colonies (Macy et al. 2000; Maggio-Price et al. 1998).

Conclusion

Microbial infections of GEM present many new concerns and challenges to the scientist using these animals, the diagnostician, and the colony manager. Genetically engineered mice may develop susceptibilities to microbial agents or novel manifestations of microbial diseases. As a result, infections may alter the phenotype of GEM and confound interpretation of results and conclusions about the functions of mutated genes. These novel host-microbe interactions have led to the development of new animal models for disease, discovery of new pathogens, and new challenges associated with control and prevention of microbial infections in GEM.

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