Genetic analysis of innate resistance to mouse cytomegalovirus (MCMV)

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Abstract

Innate immunity is inherited and is, therefore, particularly susceptible to analysis by classical genetic methods. The ‘phenotype first’ approach has already revealed the principal receptors of the innate immune system as well as several essential signalling intermediates. It has recently emerged that innate resistance to mouse cytomegalovirus (MCMV) infection depends upon a large number of host genes with non-redundant functions; hence, random germline mutagenesis frequently causes susceptibility to this pathogen. Approximately one in 30 pedigrees derived from N-ethyl-N-nitrosourea-mutagenised progenitors bears a recessive mutation that disrupts resistance to MCMV. Moreover, many of the genes required for resistance to MCMV will undoubtedly prove to have broad roles in immunity, creating resistance to many other microbes. The forward genetics approach offers an excellent opportunity to identify many of the key components of the innate immune system.

THE BIOLOGICAL PROBLEM AT HAND: WHY SHOULD WE STUDY MCMV RESISTANCE?

The identification of the proteins that mediate innate immunity in mammals, and the determination of their exact functions, are key challenges in the science of immunology. A genetic approach to this challenge can now be contemplated — and has in fact been initiated. Indeed, a genetic approach may be the only approach possible. To understand fully the problem and its dimensions, it is necessary to appreciate what immunity is all about.

All multicellular organisms must resist infection and all have evolved ways of doing so. Among the most important of these is simple physical isolation: all organisms have some form of integument that demarcates them from the environment and prevents the incursion of microbes. When physical barriers to infection fail (and they are bound to fail on occasion), however, permitting microbes to gain access to the internal milieu of the host, more specific forms of immunity are called into play. An immune system must do exactly three things: it must detect microbes, it must confine or eliminate microbes and it must be tolerant to self.

Two basic types of immunity are known. The first type of immunity, generally called ‘innate’ immunity, is phylogenetically ancient. It is activated quickly in response to infection, reaching full efficacy within minutes to hours after microbes have breached the physical barriers that protect the host. Because it is based upon a limited number of germline-encoded proteins (defined below as the ‘resistome’), it is necessarily degenerate, and tends toward a ‘one size fits all’ approach to dealing with infection. The second type of immunity, generally called ‘adaptive’ immunity, is evolutionarily recent by comparison. It is activated quickly in response to infection, reaching full efficacy within minutes to hours after microbes have breached the physical barriers that protect the host. Because it is based upon a limited number of germline-encoded proteins (defined below as the ‘resistome’), it is necessarily degenerate, and tends toward a ‘one size fits all’ approach to dealing with infection. The second type of immunity, generally called ‘adaptive’ immunity, is evolutionarily recent by comparison. It is based upon a combinatorial system of receptors and binding proteins made diverse through somatic mutation or gene conversion. These receptors are developmentally selected to ignore self, while retaining the
potential to bind avidly to molecular components of microbes. Such a system evolved on at least two occasions in vertebrates but exists in no other taxa, as far as scientists are aware.

Both innate and adaptive immunity are essential to the survival of mammals. Innate immunity is probably the more important of the two systems, however, since adaptive immunity evolved in the context of a functioning innate immune system and has never gained full independence from innate immunity. Specialised cells of the innate immune system sample the environment and present antigens to the adaptive immune system. These cells also secrete cytokines and express surface molecules that activate adaptive immune cells, stimulating them to divide when a microbial challenge is at hand. Without antigen presentation and cytokine activation no adaptive response is possible. Moreover, mutations that selectively eliminate the sensing function of innate immunity (eg mutations affecting the adapter proteins that permit signalling from the microbe-sensing Toll-like receptors [TLRs]) or eliminate innate immune cell lines (eg natural killer [NK] cells or neutrophils) are probably more severely immunocompromising than mutations that eliminate adaptive immunity altogether.

As innate immunity is inherited immunity, it is particularly susceptible to analysis by classical genetic methods. As innate immunity is actually required only under conditions of infection, mutations that impair it are generally conditional (although some proteins may have dual function and, in Drosophila, Toll — a protein that is required for innate immune sensing — has been co-opted for use in development as well). It is known that in humans, premature death due to infection is more heritable than premature death by any other cause — this despite the de facto environmental character of infectious pathogens. This implies that a large number of genes are required for robust protection against infection. But how large a number is it?

What proteins do the genes encode? Moreover, how do these proteins work together with one another to confer the relatively complex effect that is recognised as resistance? These are the questions that were posed in the opening paragraph of this review. To solve them, a focused approach is required. It is best to study mechanisms that offer resistance to a single infectious organism, confident in the assumption that the resistome for this single organism will disclose many of the proteins present in the ‘universal resistome’ — that is, the set of genes that confers resistance to all microbes. Knowledge of the universal resistome would be helpful in several ways.

First, innate immunity evolved to protect the host against small inocula of microbes, before extensive replication threatens the survival of the host. But it is imperfect and sometimes fails. When it does, overwhelming bacterial infection (sepsis) or viral infection is the result. In such circumstances, it may be considered that failure usually has a genetic cause, foreknowledge of the defect, therefore, might permit quick application of the correct countermeasure.

Secondly, and more broadly speaking, innate immune failure is the ultimate cause of all microbial disease. Since there are no universal pathogens, innate immunity is capable of eliminating all microbes in one species or another. When a particular microbe (eg smallpox) is virulent in a given host (eg humans), it is virulent because the host lacks innate protective mechanisms that make the organism non-virulent. Understanding the weak points in our armour against virulent pathogens might one day allow them to be dealt with effectively.

Thirdly, innate immunity is but one form of homeostasis. It is constantly at the ready, but is actually called upon only when the host needs it. Most of the genes that serve innate immunity are probably dispensable, absent specific circumstances (ie infection). In fact, most of the genes in the human genome may be conditional. The opportunity to identify innate
immune genes for what they are is an irresistible lure, and may tell much about what genetic methods might disclose in other systems.

Finally, the repertoire of genes that mediate innate immunity probably contains many proteins that mediate disease as well: specifically autoimmune disease. As previously commented, it may be appropriate to speak of ‘innate’ and ‘adaptive’ autoimmunity, since some autoimmune disease may be caused by failure of innate immune regulation.

MCMV AS A MODEL PATHOGEN AND SCREENING TOOL

The single pathogen of mice that has been chosen for study is mouse cytomegalovirus (MCMV). A large (230 kilobase, 170 gene) beta-herpesvirus, MCMV is most closely related to human cytomegalovirus (HCMV) and, in some respects, is a good model for the human pathogen. It has broad tissue tropism and grows to high titre in the salivary glands, liver and spleen of immunocompromised mice. It is transmitted by biting and is prevalent in the wild, infecting up to 90 per cent of wild mice. Of considerable importance to the mechanisms by which MCMV is detected, bidirectional transcription of the viral genome probably leads to the formation of abundant double-stranded (ds) RNA within infected cells, which can be detected via the TLR3 → Toll/IL-1 receptor domain-containing adaptor inducing interferon-beta (TRIF) pathway. Moreover, the viral genome is rich in CpG dinucleotides; during the rapid process of viral replication within cells, these dinucleotides remain largely or entirely unmethylated. The virus can undergo a lytic cycle, but can also remain latent through a process that is poorly understood.

Both innate and adaptive resistance mechanisms are required to cope with MCMV. If innate immunity is defective, mice may die of MCMV infection before an adaptive immune response develops. If adaptive immunity is absent (as in a mouse with severe combined immunodeficiency), innate immunity can contain the infection for a period of weeks; however, the virus will then escape innate confinement mechanisms through mutation and selection, eventually overwhelming the host.

The key cell required for innate immune defence against MCMV is the NK cell, which eliminates infected cells by producing perforin, interferon-gamma and other effector molecules. Mutations that affect NK granule formation or transport, such as the beige allele of the Lyst gene, cause hypersusceptibility to MCMV by impairing NK cell effector function. Mutational deletion of Ly49H — a gene encoding an NK-activating receptor that signals via the adapter protein DAP12 — eliminates the NK sensing function and also causes hypersusceptibility to MCMV. Ly49H senses the virally encoded protein m157, a distant homologue of MHC class I molecules. It is this protein that is usually found to have undergone mutation when the virus escapes from innate immune suppression in a SCID environment.

In most laboratory strains of Mus musculus, Ly49H does not exist (ie the locus has been deleted). C57BL/6 mice have a functional version of the Ly49H gene. On the basis of this difference, the cmv1 locus was positionally cloned. Because the C57BL/6 strain is normally quite resistant to MCMV, tolerating inocula more than 50 times larger than those tolerated by BALB/c mice, it is an excellent strain in which to examine resistance mechanisms. Such work has already begun, with the discovery that components of the TLR signalling pathways are required for MCMV resistance in vivo, and is continuing through a random mutagenesis approach, as detailed below.

In these latter studies, a particular advantage results from the shape of the MCMV dose-lethality curve, which is unusually steep. In C57BL/6 mice,
administration of $10^5$ plaque-forming units (PFU) of virus by an intraperitoneal route is rarely, if ever, fatal within seven days (a time interval corresponding to the period prior to initiation of adaptive immunity; hence, the window of time within which only innate immunity offers protection). Administration of $10^6$ PFU is uniformly fatal, however. Since a lethal dose is only slightly higher than a non-lethal dose of the virus, it may be reasoned that early recognition of the virus and suppression of viral replication makes all the difference between life and death. It is therefore possible to detect mutations that cause even relatively modest impairment of innate immunity.

Importantly, it is also possible to map these mutations. While mutations that impair immunity may be picked up and identified as transmissible even if a shallow dose–lethality slope applies for a given pathogen, mapping such mutations is virtually impossible. If a phenotype is expressed in only 20 per cent or 30 per cent of homoygotes for a particular mutation, and also expressed in 1 per cent or 2 per cent of heterozygotes, it is not possible confidently to assign a genotype to individual mice, essential for high-resolution confine of a mutation. A steep relationship between dose and lethality makes for an effective genetic screen.

**OTHER AFFERENT GENES: TLRs AND THE SUPPRESSION OF VIRAL PROLIFERATION**

With the discovery of the N-ethyl-N-nitrosourea (ENU)-induced phenotype Lps2, it was recognised that both the lipopolysaccharide (LPS) receptor TLR4 and the dsRNA receptor TLR3 share a common signalling intermediate.7,15 This protein was positionally identified7 as an adaptor protein elsewhere called Trif16 or Ticam-1.17 The knockout of Trif yielded a phenotype very similar to the Lps2 allele of Trif, but not identical — a fact that was ascribed to the presence of a modifier locus, dsRNA1, on the knockout background.19 Of particular importance to viral pathogenesis, it was shown that Trif was required for the generation of an LPS- or poly-I:C-induced type I interferon response, and also for effective containment of MCMV infection.7 Absent the normal Trif allele, MCMV was capable of killing C57BL/6 mice, while in surviving animals, very high titres of virus were measured in the spleen five days after inoculation. The proximal cause of enhanced pathogenicity was the failure to produce type I interferon in adequate amounts, as measured in the blood 36 hours after inoculation. The subsequent findings that effective containment of herpes simplex virus (HSV) infection10 and MCMV infection8
were highly dependent upon TLR9 and MyD88, confirmed the role of TLR signalling in the limitation of herpesviral proliferation in vivo. For MCMV, the sensing role of TLR3 was suspected on the basis of the earlier findings with Lps2 and confirmed by examination of knockout mice.8

Type I interferon is known to be important to MCMV containment, since targeted deletion of genes encoding either subunit of the type I interferon receptor make mice far more susceptible to infection. In the natural response to MCMV, plasmacytoid dendritic cells are the principal source of type I interferon21 and produce it in response to stimulation of TLRs 3 and 9 by dsRNA and unmethylated DNA, respectively. This interferon activates the NK cell to permit an adequate effector response.21

Other genes that participate in TLR signalling, identified in the course of screens that probe the adequacy of tumour necrosis factor production by macrophages in response to TLR stimuli administered ex vivo, have also been found to be important in limiting the proliferation of MCMV.3d, an ENU-induced phenotype in which there is defective signalling by TLRs 3, 7 and 9 (receptors for dsRNA, single-stranded [ss] RNA and unmethylated DNA, respectively), makes MCMV highly lethal to mice (Tabeta, K. et al., Nature Immunol., in press).

Interestingly, oblivious, a nonsense mutation of CD36,22 permits MCMV to grow to higher titres in vivo, although non-lethal infections are not converted to lethal ones. Identified by forward genetic screening as a co-receptor for the TLR2/TLR6 receptor complex,22 CD36 may, therefore, also participate in the recognition of a molecule derived from MCMV. Consistent with this observation, an ENU-induced mutation in TLR6 (insouciant) also seems to permit more aggressive growth of the virus. Conversely, a point mutation of MyD88 that prevents MyD88 signalling via all of the TLRs except the TLR2/TLR6 heterodimer is less permissive for virus than a knockout mutation of MyD88 (Crozet, K., unpublished data). These observations collectively suggest that the TLR2/TLR6 heterodimer, similarly to TLRs 3 and 9, contributes to the detection of MCMV, although the molecule that is recognised remains to be determined.

**EFFECTOR GENES: THOSE THAT INFLUENCE MOVEMENT OF VESICLES WITHIN THE CYTOSOL**

Mutations of the Lyst locus cause hypopigmentation of hair because melanosomes are not normally formed and inserted into the growing hair shaft. The mutation also affects coagulation and immune function, resulting from defects platelet granule function and neutrophil and NK cell function, respectively. Hence, as already mentioned, the classical beige allele of Lyst is associated with hypersusceptibility to MCMV.23

In fact, a number of mutations affecting pigmentation also have effects on viral susceptibility. Among these are some (but not all) of the mutations that cause Hermansky–Pudlak syndrome in humans: a constellation of disorders united by ocular albinism, hypopigmentation of the skin and hair, and variable problems with bleeding and immune dysfunction.24 Not all such mutations have necessarily been identified as yet, however. Moreover, some mutations that affect granule formation or function in NK cells may have no visible effect on melanosome formation nor export in melanocytes. Hence, a relatively large, and presently uncertain component, of the resistome may be represented by genes of this category.

**MANY OF THE MUTATIONS THAT CAUSE SUSCEPTIBILITY TO MCMV ALSO CAUSE SUSCEPTIBILITY TO OTHER MICROBES**

As mutations affecting MCMV susceptibility are identified by mutagenesis
and other means, it is important to see which proteins are highly specific in their ability to confer resistance to MCMV and which proteins confer broad innate resistance to other viruses and other types of microbes. Ly49H could be placed in the former category. To date, the only protein known to be recognised by Ly49H is the m157 protein encoded by MCMV, and Ly49H deficiency has no known immune consequences other than susceptibility to MCMV. By contrast, some of the TLRs (eg TLRs 3 and 9) are likely to confer very broad protection — and some TLR adapters certainly do so. Mice lacking MyD88 are highly susceptible to a wide range of viral and bacterial pathogens, as are mice with the 3d mutation.

In the present experience of the authors (described below and in Table 1), ENU-induced mutations that cause susceptibility to MCMV usually, but not always, cause co-susceptibility to vesicular stomatitis virus (VSV) in an assay system that measures the ability of VSV to cause lysis of macrophages in vitro. In the latter assay system, most of the mutations characterised to date appear to exert their effects ‘upstream’ of the type I interferon receptor. That is, they prevent effective production of type I interferon but do not render cells resistant to type I interferon, which, if added to the assay system from an exogenous source, can prevent VSV-induced lysis.

This observation suggests considerable degeneracy in the innate immune response to viruses, even viruses as different as the dsDNA beta-herpesvirus MCMV and the ssRNA rhabdovirus VSV. Somewhat less degeneracy would be anticipated if bacteria or fungi were considered as secondary screening agents. Yet, almost certainly, some proteins will prove to have universal functions in defence.

**ENU MUTAGENESIS AND THE GENOMIC FOOTPRINT OF A PHENOTYPE**
A concerted effort to identify the genetic components of the MCMV resistome has

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**Table 1**: Transmissible mutations detected by screening ENU-mutagenised mice for susceptibility to mouse cytomegalovirus (MCMV) infection. Splenic viral loads are determined five days after MCMV infection.

<table>
<thead>
<tr>
<th>Mutants</th>
<th>Phenotypic characteristics of MCMV infection</th>
<th>VSV susceptibility of macrophages</th>
<th>Type I IFN rescue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Domino</td>
<td>† Day 4 Necrotic spleen</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Goonight</td>
<td>† Day 2–3 Necrotic liver</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Solaire</td>
<td>† Day 2–3</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Slumber</td>
<td>† Day 2–3</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Jinx</td>
<td>High viral load in spleen</td>
<td>No</td>
<td>na</td>
</tr>
<tr>
<td>Warmflash</td>
<td>Moderate viral load in spleen</td>
<td>Yes</td>
<td>nd</td>
</tr>
<tr>
<td>May Day</td>
<td>† Day 3</td>
<td>nd</td>
<td>na</td>
</tr>
<tr>
<td>Paris</td>
<td>Exanthem</td>
<td>nd</td>
<td>na</td>
</tr>
<tr>
<td>Moneypenny</td>
<td>† Day 6</td>
<td>No</td>
<td>na</td>
</tr>
</tbody>
</table>

† = death  
IFN = Interferon  
nd = Not determined  
na = not applicable  
VSV = Vesicular stomatitis virus
recently been undertaken in the authors’ laboratory. Up until now, this has involved the phenotypic analysis of approximately 11,300 G3 mutant mice derived from ENU-mutagenised progenitors. Animals homozygous for randomly induced germline mutations are challenged with MCMV using an inoculum of $10^4$ PFU per mouse: a dose calculated to spare all normal animals any visible signs of infection. Animals that die or show evidence of sickness within five days are considered compromised, and an attempt is made to recover the causal mutation from the sire and dam. In this manner, 31 mutations have been recovered and, to a single significant figure, mutations are identified in approximately one of every 60 pedigrees examined. Because pedigrees of small size are constructed (six G3 mice are examined, three derived from each of two G2 females and from one G1 male), it may be calculated that approximately half of the mutations capable of causing the phenotype are actually retrieved. Therefore, about one pedigree in 30 actually has a mutation that causes immunocompromise.

The genomic footprint of a phenotype: the set of nucleotides ‘at risk’ in the sense that their alteration will create the phenotype in question

Direct sequencing estimates by Concepcion et al. and also by the present authors (unpublished data) indicate that ENU modifies approximately one nucleotide pair per million in haploid genomic DNA. This corresponds to the production of an average of four homozygous coding changes in each G3 mouse. When a phenotype is found, it is almost always ascribable to a single mutational event: a fact that is readily demonstrated by mapping. It is sometimes considered that conclusions derived from ENU mutagenesis are somehow less certain than those derived from gene targeting, wherein a specified gene is partially or entirely deleted; however, this view is misleading to the extent that gene targeting has technical problems all of its own. Gene targeting is well known for producing cis-acting effects on neighbouring genes, for example, while point mutations induced by ENU very rarely do so. Moreover, gene targeting is inherently capable of inducing unknown genetic changes. There is no assurance that every nucleotide in an ES cell line is preserved, matching those of the parent mouse with perfect fidelity. On occasion, the phenotype that is observed when a gene is knocked out may not actually emanate from the targeted locus; yet this would not normally be detected because phenotypes are not mapped after a knockout is created, they are merely assumed to be caused by the targeting event.

Among the many virtues of a forward genetics approach, one may calculate target size long before saturation has been reached. In the situation considered here, the ‘target’ is the collection of genes encoding proteins with non-redundant functions in protection against MCMV infection. How many genes are involved? How far must one go to find 90 per cent of them?

Every phenotype, however it is defined, has a ‘genomic footprint’ encompassing all of the nucleotides that are ‘at risk’ to cause it, ie those nucleotides that will, when changed by mutation, yield the phenotype. Hence, given identical protocols for mutagenesis, it is possible to calculate the relative size of the genomic footprint of any phenotype. Taking account of degeneracy, the genomic footprint (number of mutable sites) capable of yielding gait ataxia is estimated to be approximately 19,400 nucleotides; for inner ear phenotypes, the figure is 10,400 nucleotides. These numbers were derived from an analysis of 1,036 pedigrees, constructed to yield 80 per cent capture of G1 mutations in homozygous form among the G3 population (an average of 6.25 progeny per G1/G2 pair and an average of 2.8 G2 females produced per G1 male).

In the study in question, pedigree construction was designed so that approximately 80 per cent of mutations were captured in homozygous form per
pedigree, rather than 49 per cent of mutations, as is the case in the present authors’ work. Among 1,036 pedigrees, 11 instances of mutations producing ataxia and six instances of mutations producing inner ear phenotypes were observed. This implies that a total of 13.75 instances of mutations producing ataxia and 7.5 instances of mutations producing inner ear phenotypes may have existed.

Since it can be calculated that there are 62 mutations that cause MCMV susceptibility among approximately 1,883 pedigrees, as compared with 13.75 mutations that cause gait ataxia among 1,036 pedigrees, it can be estimated that the number of mutable sites capable of yielding MCVM susceptibility is approximately 2.48 times larger than the number of mutable sites capable of yielding gait ataxia. Corrected for degeneracy and for the tendency of ENU to produce A–T substitutions more frequently than G–C substitutions,27 the number of target nucleotides is approximately 48,000.

These nucleotides are parcelled among an indeterminate, but estimable, number of target genes. Assuming minimal allelism in the present authors’ existing collection — and taking account of the calculations of Kile et al.,28 who studied lethality caused by ENU-induced mutations within a restricted and well-defined genomic interval — the authors have estimated that 290 genes comprise the MCMV resistome.25 This number will be refined as tests of allelism among the target genes are performed.

HOW FAR WE NEED TO GO
All phenotypes approach saturation with approximately the same kinetics. As already mentioned, ENU creates about one base pair change per million base pairs of haploid DNA. Each nucleotide has, approximately, a one-millionth chance of being changed by ENU and, regardless of its size, the genomic footprint of a phenotype is eroded with the same half-life until complete saturation, corresponding to the mutation of every target nucleotide, is reached.

Long before saturation is approached at the level of DNA, it is approached at the level of target genes, each of which contains many nucleotide targets, any one of which can yield the phenotype as a result of modification. Little is known about the manner in which the nucleotide targets are parcelled into target genes, however. If a ‘flat’ distribution of target size obtains (ie every gene target has an equal number of equally vulnerable target nucleotides and hence an equal probability of being mutated to yield the phenotype in question), saturation is approached far more rapidly than if a highly skewed distribution obtains, with a preponderance of genes having only a few target nucleotides and a very small number of genes having the majority of target nucleotides.

In an effort to address the frequency distribution of nucleotide targets within genes, Rinchik et al.29 examined a defined genomic interval on chromosome 7 in 4,557 pedigrees hemizygous for ENU-induced mutations that caused lethal phenotypes as well as visible phenotypes. A total of 16 lethal mutations were observed, and these fell into six complementation groups (presumed to be genes). The 16 lethal mutations were distributed among the six complementation groups as follows: 6, 3, 3, 2, 1 and 1. A total of 15 non-lethal, visible mutations were also observed, and these fell into four complementation groups. The 15 visible mutations were distributed among the four complementation groups as follows: 7, 5, 2 and 1. Taking all detectable phenotypes as equivalent to one another, the frequency distribution of mutations at ten loci would thus be: 7, 6, 5, 3, 2, 2, 1, 1 and 1. This distribution might seem to suggest variability of nucleotide target frequency within target genes; however, as emphasised by Rinchik et al., these data do not permit the conclusion that target sizes are non-uniform. Seven events are not significantly different from one event
when observed within a sample of 4,557 trials. Moreover, the total number of targets (lethal or visible) within the interval in question remains unknown. A still larger number of pedigrees might disclose more complementation groups (gene targets), some having very few mutable sites, and might give a true indication of skewed target nucleotide distribution among genes and even permit derivation of an equation describing this distribution.

Kile et al. also presented data consistent with uniform target nucleotide distribution, this with regard to the lethality phenotype, occurring in a separate genomic interval on chromosome 11.28

For an innate immune phenotype (impairment of tumour necrosis factor bioactivity produced in response to stimuli capable of activating seven of the TLRs), approximately 20,000 G3 mice derived from approximately 3,300 pedigrees were examined with the observation of 11 mutations, parcelled into ten genes. Five of 22 ‘known’ target genes were struck in the exercise, suggesting that approximately 23 per cent phenotypic saturation was achieved. This observation also is consistent with uniform target nucleotide distribution.

It bears emphasis that, while no single dataset permits the conclusion that target nucleotide distributions are non-uniform among genes, this merely reflects the fact that the datasets are rather small. It is almost certain that non-uniformity applies, given large disparities between genes in terms of size and, presumably, in terms of the ability to tolerate mutation without yielding a phenotype. Assuming that approximately 300 genes comprise the MCMV resistome, however, and taking as a first estimate the notion that a similar number of nucleotide targets are present in each of them, approximately 220,000 mice would need to be examined to achieve 90 per cent phenotypic saturation (defined as the modification of nucleotide targets within 90 per cent of genes capable of yielding the phenotype).

To the extent that target size distribution among genes is skewed, a larger (perhaps far larger) number of mice would need to be examined to achieve the same degree of saturation. The performance of truly large-scale mutagenesis, involving hundreds of thousands of mice, will permit the first real insight into the size distribution of target genes.

LIMITATIONS OF GERMLINE GENETICS, OTHER APPROACHES AND THEIR LIABILITIES

Many methods are routinely used to study innate immunity. None has produced as much insight as the forward genetics approach. While gene targeting is a more frugal means of examining the role of paralogous proteins than random germline mutagenesis once the prototypic protein has been identified, initial insight does not often flow from this hypothesis-driven approach. It must be recalled that the function of the mammalian TLRs was first solved by the positional cloning of a spontaneous mutation, and that this was achieved by following a striking and promising phenotype: LPS resistance.30

Pure biochemical methods have yielded gains, but have liabilities in the study of innate immunity — the foremost being that they do not disclose the importance of pathways in intact organisms. Methods entailing overexpression of candidate proteins have yielded a number of dramatic errors of interpretation, whether a phenotypic change or evidence of contact between different proteins within cells is sought. A genetic strategy, forged by gene targeting or by random mutagenesis, is always to be preferred. Moreover, because the innate immune system is always on the alert, gene expression studies provide little insight into its hard-wired components, absent informative mutations.

To some extent, given the dynamic range of the assays that may be applied to MCMV phenotyping (for example, measuring splenic viral titre, which may vary over six orders of magnitude in...
normal mice compared with the most severely immunocompromised mutants), it will surely be possible to determine whether different mutations lie in separate pathways or in common pathways by creating compound homozygotes and assaying susceptibility. Using mutant forms of the pathogen which can grow only in immunodeficient hosts, it may be possible to infer which viral genes are directed toward the defeat of specific host resistance mechanisms. Nevertheless, it must be admitted that a pure genetic approach is best at disclosing components, rather than showing how they are assembled. If presented with the essential elements of a watch, it does not necessarily follow that one could say how they fit together. Yet without knowing the components, one has nothing at all, and genetics will surely provide the foundation for much of the understanding that is to come.

References


