of Terry Speed who spends half his time at the Walter and Eliza Hall Institute in Melbourne as a mathematical geneticist: he has a T shirt saying 'statistics means never having to say you are sure'! We may never be sure.

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Genetic resistance to smallpox: lessons from mousepox

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Abstract. There is increased interest in understanding protective immunity to smallpox for two principal reasons. First, it is the only disease that has been successfully eradicated using a live virus vaccine and, second, there exists a potential threat of intentional or unintentional release of variola virus, the causative agent of smallpox. Although mortality rates associated with smallpox were as high as 40%, a significant subset of those infected recovered. The basis of susceptibility or resistance, and the immune parameters associated with recovery, are still unknown. Animal models of poxvirus infections are being employed to understand what constitutes an effective host response. Ectromelia virus is closely related to variola virus and it causes a disease similar to smallpox in mice. This model is well established, resistant and susceptible strains of mice are defined and four genetic loci associated with resistance have been identified. Susceptibility to infection and disease severity is also influenced by virus immune evasion strategies. The outcome of infection is clearly dictated by several factors including host and viral genes, both of which influence the immune response. Here we present data on one virus-encoded immune modifier and its effect on the functions of two host genetic loci associated with resistance.

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Host response to viral infection and determinants of disease outcome

The host response to viral infection may be divided into two distinct but complementary and interactive parts. The first comprises a series of rapidly acting antimicrobial mechanisms, including natural killer (NK) cells, which form the innate phase of the immune response. These mechanisms protect the host in the earliest stages of infection. They prevent most infections from becoming established and allow time for generation of the slower adaptive immune response, including cytotoxic T lymphocytes (CTLs), that are antigen-specific and which establish immunological memory. The innate response which is comprised of a range of soluble factors and various leukocyte subsets can profoundly influence and direct

the type of adaptive response that is generated and is therefore critical in determining the outcome of a viral infection. Disease severity and outcome are influenced by factors such as virus strain, virus immune evasion strategies, dose and route of infection. The outcome of infection is therefore dictated by several factors including host and viral genes, both of which profoundly influence the immune response.

Protective immunity to smallpox

Smallpox was one of the biggest human scourges, resulting in mortality rates of up to 40% in some populations. However, a significant subset of the infected population recovered. The basis of susceptibility and resistance, and the immune parameters associated with recovery, is not known as the virus was eradicated more than 25 years ago. Despite the success of the smallpox eradication program, there remains considerable fear that variola virus (VARV), the causative agent of smallpox, or other related pathogenic poxviruses such as monkeypox (MPXV) could re-emerge and spread disease in the human population. The increased interest in understanding protective immunity to smallpox is due not only because of the potential threat of a bioterrorist attack (Henderson et al 1999) but it is the only disease known to humankind that has been successfully eradicated with a live virus vaccine. As such, very useful information on immunity and resistance to disease may be gleaned from the study of smallpox.

Smallpox, mousepox and genetic resistance

Since VARV has a restricted host range and is known to infect only humans, closely related orthopoxviruses, such as MPXV and ectromelia virus (ECTV), have been used extensively in animal models to elucidate pathogenesis and immune response to infection. Currently, the best surrogate for VARV in a small animal model is ECTV, as it is infectious at very low doses, has a restricted host range, encodes a similar repertoire of immune evasion proteins and causes severe disease (mousepox) with high mortality rates (Esteban & Buller 2005, Fenner et al 1988, Seet et al 2003). Further similarities between smallpox and mousepox include virus replication and transmission, cytokine responses and many aspects of pathology (Esteban & Buller 2005). Thus, to understand the genetic basis of resistance and susceptibility to smallpox in humans, we have used mousepox, where resistant and susceptible mouse strains are well defined and at least four genetic loci associated with resistance have been mapped.

Mousepox: a model for study of virus–host interactions and smallpox

ECTV is a natural mouse pathogen that has co-evolved with its host. Inbred strains of mice are resistant or susceptible to infection with ECTV. The virus

causes an acute disease characterized by generalized viral spread and pathology. Death usually occurs due to extensive necrosis of major organs, in particular the liver and spleen, as a result of massive virus replication (Fenner 1948). Both MHC and non-MHC genes determine resistance or susceptibility to mousepox. Strains such as A/J (H-2a), BALB/c (H-2d) and DBA/2 (H-2d) exhibit high mortality (100%) to mousepox while C57BL/6 (H-2b), C57BL/10 (H-2b) and 129 (H-2b) strains have very low mortality, limited pathology and are classified resistant. Wild mice show variable susceptibility to mousepox (Buller et al 1986). The mousepox model has been utilized extensively to study virus–host interactions, genetic resistance to disease and viral immunology (O'Neill et al 1983, Buller et al 1986, Brownstein et al 1992, Karupiah et al 1993, 1996, 1998, Brownstein & Gras 1997, Mullbacher 2003, Chaudhri et al 2004, 2006, Panchanathan et al 2005, 2006, Tschärke et al 2005). Since mousepox is a model of a *natural infection*, it is a powerful tool for understanding the immune response. It not only provides a useful model for smallpox but also for other generalized viral infections.

Immunity to ECTV infection: Roles of innate and adaptive immune responses

The resistant strains of mice, such as C57BL/6, generate a strong T helper 1 (Th1)-type cytokine response (i.e. interleukin [IL]2, γ interferon [IFN γ] and IL12) and potent NK cell and antiviral CTL responses whereas these responses are either delayed and weak or lacking in susceptible strains of mice (Chaudhri et al 2004). We have defined key immune parameters in C57BL/6 mice that are important for recovery from mousepox. These include the effector functions of NK cells, CD4⁺ and CD8⁺ T lymphocytes, B lymphocytes and antibody, macrophage subsets, nitric oxide and IFN α , β and γ (Chaudhri et al 2004, 2006, Karupiah et al 1996, 1998, 1993, Panchanathan et al 2005, 2006, Ramshaw et al 1997). Perforin, granzymes and IFN γ are necessary for the effector functions of CD8⁺ T cells and NK cells in the control of this virus (Karupiah et al 1993, Mullbacher 2003, Panchanathan et al 2006, Ramshaw et al 1997).

The role of antibody in recovery from a primary ECTV infection was only recently established (Chaudhri et al 2006, Fang & Sigal 2005). We have previously reported that in C57BL/6 mice, which are normally resistant to mousepox, the absence of CD4⁺ T cells resulted in ECTV persistence for extended periods (Karupiah et al 1996) and the animals eventually succumb to disease. The antiviral CTL response in mice lacking CD4⁺ T cells was suboptimal, suggesting that virus persistence maybe the result of a defective CTL response. However, in contrast to the C57BL/6 wild-type mice, antiviral CTL activity in these mice persisted even in the late stages of infection (Karupiah et al 1996). Notwithstanding, the CTL were insufficient to clear virus. Since CD4⁺ T cell help is also crucial for antibody production (MacLennan et al 1997, Parker 1993), we hypothesized that virus

TABLE 1 Genetic loci that control resistance to mousepox in C57BL/6 mice

Designation	Location	Locus	Reference
<i>Rmp1</i>	Chromosome 6	Natural killer cell complex	Brownstein & Gras (1997), Delano & Brownstein (1995)
<i>Rmp2</i>	Chromosome 2	Complement component C5	Brownstein & Gras (1997)
<i>Rmp3</i>	Chromosome 17	MHC (H-2) complex	Brownstein et al (1992)
<i>Rmp4</i>	Chromosome 1	Selectin gene complex	Brownstein & Gras (1997)

persistence in these animals might be due to defective antibody response. Indeed, mice deficient in B cells (Kitamura et al 1991) succumbed to mousepox between 3–4 weeks post-infection although they appeared to keep the infection under check during the first two weeks. In many respects, the kinetics of virus replication and outcome of infection in B cell deficient mice was similar to mice deficient in CD4⁺ T cells (Chaudhri et al 2006). Our data show that mice deficient in CD8⁺ T cells or CD8⁺ effector function die early in infection whereas those deficient in B cells or antibody production die much later, indicating that B cell function becomes critical after the effector phase of the CD8⁺ T cell response to infection subsides. In mice lacking B cells or antibody, ECTV persists and the host succumbs to disease, despite the generation of normal CD8⁺ T cell responses (Chaudhri et al 2006). The importance of antibody in a primary infection had not been previously appreciated.

Genetic resistance to mousepox

In mice, there are at least four loci known to confer resistance against mousepox (Table 1). The resistance to mousepox (*Rmp1*) locus on chromosome 6 maps to the NK gene complex (NKC) (Brownstein & Gras 1997, Delano & Brownstein 1995). The *Rmp2* locus on chromosome 2 maps near the complement component C5 gene (Brownstein & Gras 1997). *Rmp3* is also gonad-dependent and is linked to the MHC (H-2) on chromosome 17 (Brownstein et al 1992). Finally, the *Rmp4* locus, on chromosome 1, maps to the selectin gene complex (Brownstein & Gras 1997). Only *Rmp1* and *Rmp3* will be briefly discussed here.

The *Rmp1* locus

The critical role NK cells play in the control of infection with viruses is well-recognized (French & Yokoyama 2003). In the murine cytomegalovirus (MCMV) model, susceptibility to lethal infection can be overcome by a dominant allele at

the *Cmv1* locus in the C57BL/6 mouse. *Cmv1* maps to the NKC on chromosome 6 and controls early replication of MCMV via regulation of NK cells. It is now established that *Cmv1* is Ly49H, an NK cell-activating receptor (Arase et al 2002, Forbes et al 1997). The NKC in the BALB/c mouse strain lacks some activating receptors, including Ly49H and is therefore susceptible to MCMV whereas the C57BL/6 strain is resistant. The generation of BALB/c mice congenic for C57BL/6 NKC (designated BALB/c.B6.Cmv1^r) (Scalzo et al 1999) has been important to definitively establish that *Cmv1*^r is Ly49H. More recently, we have used the BALB/c.B6.Cmv1^r mice in the ECTV model to study the role of the NKC in conferring genetic resistance to mousepox.

The *Rmp1* locus on chromosome 6 controls replication of virus in the spleen and liver. It maps to the NKC (Brownstein & Gras 1997, Delano & Brownstein 1995) and this is consistent with the known importance of NK cells protection against infection (Brownstein & Gras 1997, Delano & Brownstein 1995, Karupiah et al 1996). We have recently shown BALB/c.B6.Cmv1^r mice displayed increased resistance to ECTV infection compared with wild-type BALB/c mice, which showed uniform mortality. The increased resistance of BALB/c.B6.Cmv1^r mice was completely abrogated when NK1.1 cells were depleted, indicating that the effect was due to NK cells. In addition, we have preliminary evidence that treatment of ECTV-infected BALB/c.B6.Cmv1^r mice with monoclonal antibodies to specific Ly49 family of proteins significantly increased viral load in the spleen and liver but not as much as titres in mice depleted of NK1.1 cells. We predict that the activity of *Rmp1* may be attributed to at least two or more NK cell-activating receptors.

*The *Rmp3* locus*

Rmp3 is linked to the H-2 complex (Brownstein et al 1992) and its function is expressed through CD8 T cells, which are essential for recovery from primary ECTV infection (Karupiah et al 1996, O'Neill et al 1983). The resistant strains of mice, such as C57BL/6, generate a strong Th1 cytokine response and potent antiviral CTL responses early in infection whereas in susceptible strains of mice such as A/J and BALB/c, these responses are delayed and weak or completely lacking (Chaudhri et al 2004). In general, mice of the H-2^b haplotype (C57BL/6, C57BL/10 and 129) are resistant, mice of the H-2^d/H-2^a haplotype (BALB/c, DBA/2, A/J) are highly susceptible and those of the H-2^k haplotype (C3H, CBA) tend to be intermediate in susceptibility. We have preliminary evidence that C57BL/6 mice lacking MHC class I D^b are highly susceptible to mousepox, suggesting that the function of *Rmp3* may be attributed to, or expressed by, D^b. Although the immunodominant CD8⁺ T cell determinant to ECTV is restricted by K^b (Tscharke et al 2005), mice deficient in this class I molecule were able to

overcome infection with ECTV unlike the D^b GKO mice. Mice lacking both K^b and D^b molecules were highly susceptible to mousepox.

Influence of poxvirus-encoded host response modifiers to disease resistance

Viruses have evolved numerous mechanisms to evade detection and destruction by the host immune system. Orthopoxviruses have devised novel strategies, which directly target molecules of the immune system and can therefore influence the activities of NK cells and CD8 cells. ECTV, like VARV, encodes molecules that target the host apoptotic pathway, complement cascade, semaphorins, chemokines and cytokines. Of the viral proteins that target host cytokines, those which influence NK and CD8⁺ T cell responses are viral IFN γ binding protein (vIFN γ bp), vIFN α / β bp and vIL18bp. Indeed, susceptible BALB/c mice infected with a deletion mutant ECTV lacking vIFN γ bp (ECTV-IFN γ bp^{-/-}) were able to effectively clear virus and recovered, unlike mice infected with wild-type ECTV, which succumbed to mousepox. The absence of vIFN γ bp resulted in increased host IFN γ production that allowed the BALB/c mice to generate elevated NK cell and antiviral CD8⁺ T cell responses (unpublished data). These data are consistent with the idea that the full expression of genetic resistance by the host to virus infection is clearly affected by virus-encoded immunomodulatory genes.

Summary

We have used the mousepox model as a model for smallpox to understand the roles and mechanisms of some host and viral genes in conferring resistance to disease and show that both viral and host genes profoundly influence the outcome of infection.

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DISCUSSION

Casanova: What is your strategy for identifying the NK susceptibility gene?

Karupiah: We're going to use the same strategy that Tony Scalzo and Wayne Yokoyama used with the mouse cytomegalavirus model, which is to use antibodies specific for the Ly49 family of molecules. We have done some work with Ly49 H and D. We think it is one of them, but we think there is more than one: it is not a single gene.

Casanova: Do you think it could be a susceptibility haplotype with several genes nearby involved?

Karupiah: We have some evidence that it could be both Ly49 H and D.

Footte: It seems that B6 mice are particularly resistant to many infectious diseases, such as malaria and leishmaniasis. Everyone seems to take the same approach to this, looking at cytokine profiles. Inevitably, they reach a place where they find that cytokine profiles and many of the immunological phenotypes they are measuring are almost irrelevant to the phenotype that is being looked at. You have a couple of instances where you find exceptions to this rule, but by and large there seems to be a discord between cytokine profiles and differences in susceptibility.

Karupiah: Taking just one cytokine as an example, we know that IFN γ is critical in the three different strains we have looked at. The clue to why it is important perhaps comes from the viral-encoded IFN γ binding protein. I agree with you that some of the cytokines that we think are important might not matter, but there are some that are critical.

Footte: There are congenics available, especially from the chick group where they have the congenics for some of the cytokine responses. It would be interesting to challenge these to see whether the cytokine changes are important at the functional level. This is different to knocking the gene out.

Hume: Along the same lines, it is self-evident that an appropriate T cell activation response is required. In looking at the cytokine profiles, you are studying what Peter Doherty called the interface of microbiology, immunology and pathology, which is mythology. You are downstream of the consequences of the initial recognition of the pathogen. In the early work on bacterial susceptibility loci, on this same sort of cross, you could tell that the outcome of the infection was determined

in the first 30 min, depending on whether BCG was localized to the initial site of injection or disseminated, for example. Is there a cell autonomous difference in the primary recognition and response to the pathogen? In other words, do macrophages take it up to the same extent and induce the same early response genes? If those are different, everything else follows.

Karupiah: The only thing we know about the macrophage difference seems, at least *in vivo*, to be at the level of the Kupffer cells in the liver. One level of resistance seems to be whether Kupffer cells in the liver can support replication. We know that this happens in susceptible strains.

Hume: If that were to be the case, wouldn't it make sense to gene profile the precise phenotype of Kupffer cells between the resistant and susceptible mouse strains. What is the primary difference in determining the outcome of infection? At what stage in the infection process can you tell that the animal is going to fail to control the replication of the virus?

Karupiah: It is not as simple as whether the macrophage gets infected or not. Many responses are generated which activate the macrophage and make the activated macrophages in a resistant mouse able to control the infection, preventing spread to the hepatocytes.

Hume: Do you have evidence that macrophages from the resistant and susceptible strains are infected in exactly the same way?

Karupiah: Yes, *in vitro* and *in vivo*. I don't think it is simply at the level of macrophages. It is more complicated. Of course, macrophages are critical. About 10 years ago we used liposomes to deplete macrophages. They are not able to generate a host of responses, including the cytotoxic T cell response. I believe that macrophages, in addition to neutrophils, are the earliest players that need to be involved in some way in presentation of antigen for the generation of the cytotoxic T cell response. There are differences in the susceptible and resistant strains in terms of activation and production of not only cytokines but also activation of other cell types needed in order to have this genetic resistance expressed. I don't think it is just simply macrophages.

Hume: One question is whether a B6 nude resists a virus any better than a BALB/c nude.

Karupiah: This doesn't speed up the death process. If you infect a nude mouse, whether it is BALB/c or B6, they die about the same time. But the process can be speeded up by using a nude which doesn't have IFN γ .

Maizels: Why don't you get sterilizing immunity from the CTL population? In the resistant mouse for two weeks they seem to keep everything in bay, but what happens after this? Do the CTL decrease in frequency, or are the interference proteins from the virus able to switch off the CTLs?

Karupiah: That's not the case. Even at 35 d the CTLs are still there, and they are still cytolytic. It appears that the antibody is needed to control virus in blood. Once