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Review Article

GENETIC DETERMINANTS INFLUENCING THE RESPONSE TO INJURY, INFLAMMATION, AND SEPSIS

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ABSTRACT—The genetic background has recently been recognized as an important element in the response to injury, contributing to the variability in the clinical outcome of critically ill patients. The traditional approach to studying the genetic contribution requires the availability of families with multiple members who have experienced similar disease conditions, a situation that is nearly impossible to find in the case of trauma. Association studies looking at unrelated individuals across populations require large economic and labor-intensive efforts. Thus, a candidate gene approach has been the sole methodology used to correlate genetic variability with clinical outcome. However, this approach cannot provide a comprehensive description of a multigenic condition. Animal models are an alternative for studying the genetic contributions to variability in the response to injury. A murine model is ideal because a large set of inbred strains are available; congenic, consomic, transgenic, and recombinant strains can also be used. Employing this paradigm, we have demonstrated that the response to several stressors, such as injection of *E. coli* lipopolysaccharide (LPS) and polymicrobial sepsis induced by cecal ligation and puncture (CLP), is modified by the genetic background. The inflammatory response in mice has also been shown to be affected by sex, age, and other, nongenetic components such as diet. We have exploited the differences in response among various inbred mouse strains to map loci contributing to the inflammatory response. Fine mapping strategies allow the refinement of sets of candidate genes, which can be identified by positional cloning. Detection of genetic variation affecting the inflammatory response in murine models provides a basis for determining whether polymorphisms in orthologous human genes correlate with particular clinical outcomes from injury. Thus, discovery of these genes could impact patient care by acting as markers of a specific predisposition in humans.

KEYWORDS—Sepsis, inflammation, injury, genetics, aging, sex

INTRODUCTION

Cardiovascular disease, cancer, and trauma are the three major causes of mortality in developed countries. If these data are stratified by the age of the subject, trauma emerges as the major killer of people under 40 years of age. Part of the mortality observed after trauma is associated with the development of a secondary condition known as sepsis, which is triggered by the initiating insult. The most detrimental consequence of sepsis is the development of multiple organ dysfunction syndrome (MODS), which is generally a fatal condition (1, 2). Over 750,000 cases of sepsis are expected per year within the United States (3). Moreover, sepsis is not initiated only after a traumatic injury but may develop following other conditions, such as pancreatitis or even elective surgery. Despite technological, pharmacological, and surgical advances, therapy for sepsis remains supportive, and mortality from this condition remains the leading cause of death in noncoronary intensive care units.

The precise etiology of sepsis and MODS is not known, but these conditions are likely the result of a poorly regulated inflammatory response (4). Inflammation is a complex process modulated by several pathways and a multitude of genes. Cytokines are important mediators of the inflammatory response and, thus far, have been the best markers of this process. The improved understanding of cytokine biology and its impact on the response to injury created the expectation that cytokine regulation could be used as a possible therapy for sepsis and MODS. However, cytokine blockade treatment produced unsatisfactory results in several clinical trials, despite its great success in experimental animal models (4–6). The question that emerges is whether the human population that was tested was the best fit for the proposed therapy. Moreover, it is not clear how closely the experimental animal models resembled the human disease condition. By design, the experimental animal models that we currently use do not represent the complexity of critically ill patients. Consequently, we should avoid "short cuts" in the development of experimental animal models that replicate the symptoms observed in human patients to test different therapeutic procedures. It is also important to remember that humans are not identical but, rather, genetically very heterogeneous. Humans come in two biological sexes that are the combination of sex-specific hormones and the expression of sex-specific genes. Humans also present numerous confounding social factors, such as smoking, drinking, obesity, and stress. Thus, the characteristics of the human population enrolled in a clinical trial may be critical to the final outcome of the study.

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THE ROLE OF GENETICS IN THE RESPONSE TO INJURY

A question that continues to puzzle clinicians is the basis for the tremendous variability seen in the clinical profile of patients who encounter similar insults. We have proposed that the clinical outcome from injury is a combination of several factors including the initiating insult, the environment, and the genetic makeup of the subject (7). Two additional factors, sex and age, need to be included in this paradigm because their impact on the response to injury is clear and important. However, it is also evident that sex and age reflect the overlap between genetics and the environment. Thus, we propose that the intersection of these factors (i.e., initiating insult, environment, genetics, sex, and age) will determine a particular outcome (Fig. 1). Therefore, the variable response to injury and the failure of the clinical cytokine blockade trials may be explained, at least in part, by the genetic diversity of the human population.

The role of genetics in the response to stress has been illustrated in several scenarios. One important clinical observation is the heterogeneous response to injury observed in patients. This is particularly true when the incidence of sepsis, acute respiratory distress syndrome (ARDS), and MODS are analyzed with respect to mortality rates. These conditions are not observed in all patients experiencing similar primary stressors. Another argument stems from the frustration experienced by many clinicians when patients die of MODS after successful, routine operative procedures. Genetic determinants that may be involved in the response to injury have been associated with similar disease conditions, such as acute necrotizing pancreatitis (8).

Several approaches have been successful in mapping genes involved in a particular disease. The most common approach is traditional linkage analysis, which is not applicable to the study of a predisposition to trauma or sepsis because the limiting event does not occur in families. Moreover, these conditions are multifactorial and observed only after a stochastic initiating event, complicating any possible linkage analysis. Association studies looking at unrelated individuals across populations involve typing hundreds or thousands of markers across a large panel of subjects, a logistically complex process, and are labor intensive, expensive, and time consuming. An alternative method is the candidate gene approach in which genes of interest are analyzed to find polymorphisms correlated with the incidence of a disease. This alternative has been illustrated in the analysis of polymorphisms at the human TNF-α and TNF-β loci (9–11). These studies in humans were undertaken because polymorphisms in the promoter region of mouse TNF-α had been identified and correlated with circulating TNF-α levels and with several pathological conditions in the mouse (11). Similarly, caspase-12 polymorphisms have been associated with a hyporesponse to lipo polysaccharide (LPS) (12). Comparable surveys could certainly be developed for other components of the inflammatory response, although the success of this approach is less evident because of the multifactorial condition of sepsis. Polymorphisms of several genes, including mannose-binding lectin (13), heat shock protein 70 (14), CD14 (15, 16), Toll-like receptor 4 (17), and interleukin (IL)-6 (18), have been used to study susceptibility to sepsis. However, the success of these studies in identifying genetic markers for a predisposition to sepsis has been limited (19–21). The major concern is that candidate gene analysis would require an educated guess as to the appropriate gene to be studied. Moreover, this approach is not likely to reveal all of the several genetic determinants controlling the inflammatory response.

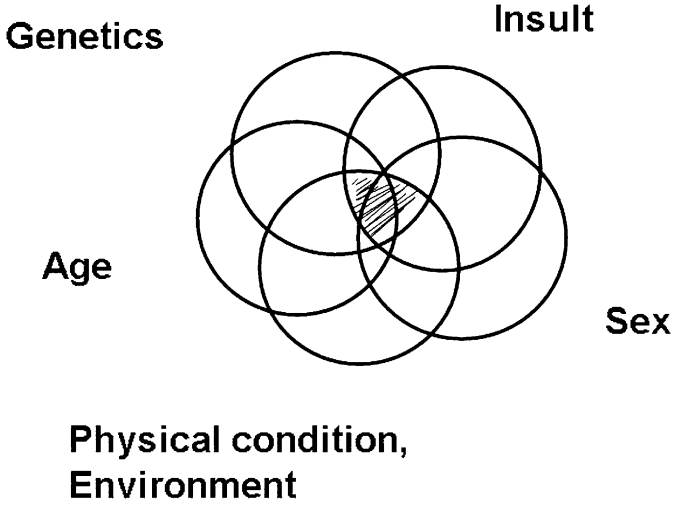

FIG. 1. **Paradigm of injury outcome.** A number of factors interact to determine the outcome of trauma in a given individual.

THE USE OF A MOUSE MODEL TO STUDY THE GENETIC CONTRIBUTION TO INFLAMMATION

An alternative means of studying the genetic contribution to the stress response is through the use of an animal model. A mouse model is particularly useful in studying the role of genetics in the response to injury because a large number of inbred strains are commercially available. If different inbred strains are maintained in an identical environment and subjected to the same insult, possible differences in their responses can be attributed to genetic differences among them. In other words, allelic variability between inbred strains may modulate the response to injury (e.g., mortality, cytokine levels). Moreover, the biological response of different mouse strains can be used to map the contributing genes. Mice have several additional advantages, including short generation time, large number of progeny from controlled crosses, and the ability to be inbred to homozygosity. Furthermore, there are several mouse resources available, such as recombinant inbred (RI), congenic, and consomic strains. Although details of the inflammatory response differ among species, the set of genes that has evolved to carry out this central function is highly conserved. Additionally, the use of mouse genetic resources to study the response to injury can help in the elucidation of particular components or pathways involved in this complex process. There is a lesson to be learned from yeast genetics. Several years ago, researchers focused on yeast to study the basic cellular processes that occur in mammalian cells. The generation of mutants with different phenotypes on basic cellular processes and the capability to clone the involved gene by

complementation have accelerated our current knowledge of different cellular processes in higher eukaryotes. Without yeast genetics, it would have been impossible to advance so rapidly in such fields as cell biology and biochemistry. Obviously, the stress response of unicellular organisms is not as complex as that observed in multicellular organisms. Moreover, sepsis cannot be studied in yeast. Thus, the mouse may be the "yeast equivalent" of modern injury biology, as this rodent displays a comprehensive response to injury and sepsis that mimics several of the symptoms observed in humans.

THE INFLAMMATORY RESPONSE IS GENETICALLY MODULATED

We have shown differences in the frequency of mortality among five inbred mouse strains after injection of *E. coli* LPS. Because these mice were maintained in identical environments and subjected to the same insult (i.e., LPS), the difference in mortality could only be caused by the genetic diversity among them (7). In addition, LPS-induced cytokine plasma levels were different among various inbred mouse strains. Whole-body pathology 24 h after LPS injection also revealed a highly significant difference in the number of infiltrating polymorphonuclear leukocytes (PMN) in liver and lung from C57BL/cJ (B6) mice as compared with A/J mice (22). Moreover, naïve peritoneal macrophages (PMϕ) isolated from different mouse strains displayed different patterns of cytokine release when challenged with LPS in culture conditions (Fig. 2), further supporting the role of genetics in the inflammatory process. Although the use of isolated PMϕs is a step toward more mechanistic studies for increasing our understanding of the intersection between the inflammatory response and genetics, there are limitations to these *ex vivo* analyses. Macrophages in culture do not perfectly mirror *in vivo* conditions. When macrophages are stimulated with LPS in culture, the cytokines that they secrete can act on the cells in an autocrine fashion at higher local concentrations than would be seen *in vivo*, thereby modifying their responses. For example, IL-10 can down-regulate TNF-α production. Thus, the levels of TNF-α produced by cultured macrophages may be underestimated because of the suppressive effect of IL-10. Moreover, systemic conditions reflect the interaction between multiple factors and cell types. Despite these limitations, the use of macrophages in culture is a powerful means of studying the components of inflammation because it allows the use of more controlled experimental conditions. For instance, isolated PMϕs have been used to study the effect of aging and diet on the inflammatory process (23).

Although LPS contributes to sepsis, the overall response to trauma is far more complex than the effects of LPS alone. Polymicrobial fecal peritonitis induced by cecal ligation and puncture (CLP) is a more intricate animal model of sepsis that better reflects the complex response of this syndrome in humans. This animal model represents the combination of three insults: tissue trauma as a result of the laparotomy; a site of necrosis caused by obstruction of the blood flow to the ligated portion of the cecum (ischemia without reperfusion); and the leakage of peritoneal microbial flora and fecal particles within the peritoneum. An advantage of the CLP model is that the severity of the initial insult can be modulated by varying the area of cecum ligated and the size or number of perforations. The severity of the response in this model can be further modified by using different resuscitation strategies or by the use of antibiotics.

We showed that CLP, using a single puncture with a 25-gauge (G) needle and single resuscitation injection of saline, caused a significant difference in the mortality frequency between male B6 and A/J mice (25), similar to our prior observations after LPS injection (7). This finding indicates that a genetic component contributes to the outcome from CLP as well. Circulating levels of IL-10 were significantly different between B6 and A/J mice after CLP and, in fact, were very similar to those observed after LPS (Fig. 3). However, no differences in TNF-α, IL-1β, or IL-6 were detected between B6 and A/J mice following CLP, which were different between these two mouse strains after injection of LPS (7). Comparable levels of hepatic PMN infiltration were observed in B6 (higher) and A/J (lower) mice after CLP and after LPS. Thus, it may be

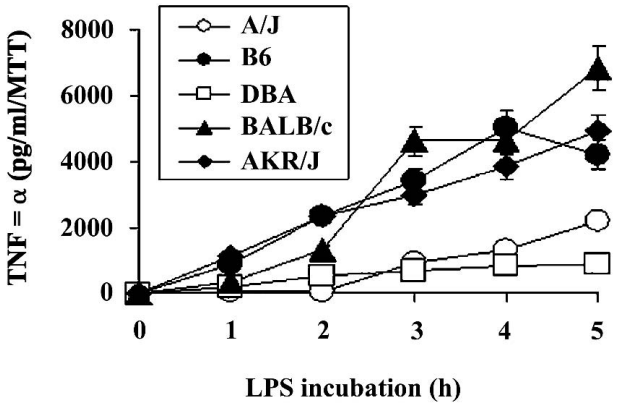

FIG. 2. **LPS-induced TNF-α levels vary among different mouse strains.** PMϕs were isolated from 2-month-old A/J, C57BL/cJ (B6), DBA/2J, BALB/cJ, and AKR/J mice. Cells were stimulated with LPS (100 ng/mL) for 1 to 5 h. The extracellular medium was collected, and TNF-α levels were determined by ELISA. Values were normalized by the number of live cells in each well (MTT assay) and expressed as pg/mL/MTT (courtesy of Dr. Virginia Vega).

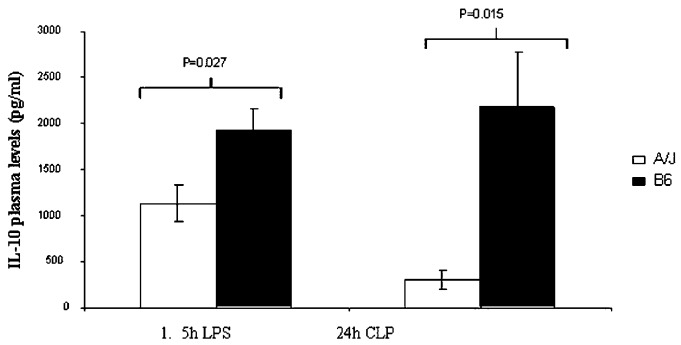

FIG. 3. **Comparison of IL-10 plasma levels after CLP or LPS in A/J and B6 mice.** B6 and A/J male mice were fasted for 16 h and subjected to CLP or injected with LPS (15 mg/kg). Plasma was obtained from blood samples collected 24 h after CLP or 1.5 h after LPS. IL-10 levels were measured by ELISA and are presented as mean ± SEM. Statistical significance was obtained by Mann-Whitney Rank Sum Test. IL-10 levels were significantly different between B6 and A/J mice for each insult.

concluded that the inflammatory response after CLP is similar, but not identical, to that induced by LPS (24), which is consistent with prior reports (25). A genetic contribution has also been noted after other types of insults. Mucosal damage and bacterial translocation after burn injury was observed to differ among various mouse strains (26). Differences were also observed in the infection rate of parasitic infections (27) and exposure to toxic agents (28). Higher mortality of BALB/c versus C57BL/6 mice was noted after mechanical (two-leg tourniquet), thermal, and radiation trauma (29). In addition, the combination of insults, such as CLP, injection of LPS, and surgical trauma (laparotomy), resulted in a response that, although not reflecting the addition of the individual insults, still conserved the difference in response among mouse strains (30).

SEX IS AN ADDITIONAL FACTOR THAT MODIFIES THE INFLAMMATORY RESPONSE

It is well accepted that women have a more active immune system than men. However, whether women as a group experience better outcomes from injury as compared with men is still controversial. Among nine retrospective and prospective clinical studies, four reports showed a worse outcome to injury (as measured by mortality) in male with respect to female subjects (31–34), one showed higher mortality in female patients (35), whereas no differences between the sexes were observed in the remaining four studies (36–39). Moreover, a higher incidence of severe sepsis (39, 40) and a higher incidence of infections have been seen in male patients (41–44). Studies using experimental animal models have provided a better understanding of the role of sex in the response to injury. In general, female rodents have shown an enhanced immunological response with respect to male animals, resulting in better survival after injury (45). The difference in response between female and male rodents has been predominantly attributed to the differing hormonal milieu of each sex. For example, a decrease in testosterone levels by either castration or pharmacological blockage has been shown to be beneficial in C3H/HeN male mice after hemorrhagic shock (46–49). Similar studies using CLP have provided evidence that female C3H/HeN mice are more immunologically competent than male mice in enduring this insult (50). However, no difference in mortality was observed between female and male C57BL/6J mice after injection of LPS (51). Secretion of cytokines by isolated peritoneal and splenic Mϕs from C3H/HeN mice after hemorrhagic shock was higher in female than male subjects, and this effect was attributed to the presence of estradiol (50, 52, 53). We have found that the inflammatory response induced by LPS can be modified by the presence of sex steroids depending on the genetic background of the mouse, which may explain some of the contradictory observations that have been reported (54). Moreover, autosomal, genetically identical individuals carrying differences in their sex alleles (e.g., female and male mice from the same strain) present with different inflammatory responses, suggesting that factors in addition to sex steroids are involved.

AGE SHOULD ALSO BE CONSIDERED IN THE DESIGN OF AN APPROPRIATE ANIMAL MODEL OF SEPSIS

Another potential component that may contribute to the disparity in the development of sepsis is age. Aging is a biological process characterized by a multitude of unfavorable changes that result in decreased homeostasis, increased incidence of age-related degenerative diseases, and death. The mechanisms underlying the alterations that occur during aging are not well understood but likely include the modulation of gene expression, a decrease in translation fidelity, an accumulation of mutations and abnormal proteins, and modifications of neuroendocrine control (20). In addition, aging is modulated by multiple factors, including nutrition, social behaviors, clinical history, and the environment. The genetic background is also likely to play an important role in the aging process, as shown by investigations using invertebrate models (55). Studies in rodents have shown a relationship between age and increased mortality following CLP (56). A compromised immune system has been observed with age, which is likely responsible, at least in part, for the increased incidence of inflammatory and autoimmune diseases and susceptibility to bacterial infections observed in older individuals (57–59). In addition, a lowered degree of inflammation, illustrated by the constitutive presence of TNF-α, IL-6, soluble TNF-α receptor, and acute-phase proteins, has been reported during aging (60–62). An increase in oxidative stress is also observed in the elderly, which is caused by an excess of oxidant production over antioxidant levels (63). Age-related changes in the immune system include a reduction in clonal expansion and a decrease in the function of antigen-specific T and B cells and antigen-presenting cells (57, 64, 65).

A chronic stress that affects macrophage function has also been suggested as part of the aging process (66). LPS stimulation of white blood cells isolated from aged volunteers showed a lower production of TNF-α and IL-1β than cells from younger individuals, probably because of monocyte dysfunction (67). A reduction in LPS-induced TNF-α in PMϕs derived from older mice in comparison with younger animals has also been reported (68). Similar results were obtained using PMϕs derived from aged rats, which had reduced superoxide production and TNF-α secretion in response to interferon-γ (69, 70). A decrease in the number of alveolar macrophages is observed in older rats, resulting in decreased pathogen clearance (71). Moreover, the production of reactive oxygen species by alveolar macrophages from older rats is higher than that observed in younger animals, which correlates with increased lung damage after bacterial infection (72). In addition, the function of hepatic Mϕs or Kupffer cells is altered during aging, showing a relationship with the increased susceptibility to sepsis after trauma and infection in older people (73). A decrease in phagocytosis with age has been associated with alterations of cytoskeleton components (74), impairment of respiratory burst activity (75), and decreased expression of cell surface proteins (76–78) and transcriptional factors (79).

We observed an inverse linear relationship between TNF-α and IL-10 levels and age. This inverse linear relationship was

not observed for IL-6 or IL-1β, although their levels were also affected by age. In general, cells derived from younger animals showed higher cytokine levels than cells isolated from older mice (23). These observations are consistent with prior studies that indicated a decline in cytokine production during aging (68, 69). Because chronic inflammation has been observed in older individuals, the reduced cytokine production by Mϕs from older mice may be related to an effect of tolerance or attenuation. Alternatively, the decrease in cytokine production with age may be related to a reduced capacity of LPS stimulation. In fact, a reduction in CD14 on the plasma membrane of PMϕs isolated from the older, 18- and 24-month-old mice in comparison with the juvenile, 2-month-old mice was observed (23). Thus, the lower production of cytokines with age may be related to a reduced capacity to transduce the LPS signal, secondary to reduced CD14 surface levels.

GENES REGULATING THE INFLAMMATORY PROCESS CAN BE IDENTIFIED BY MOUSE GENETICS

Mouse models have proven successful in the identification of modifier genes involved in many diseases (80, 81). Mapping of quantitative trait loci (QTL) allows us to identify genes that modify the response to injury, thus determining a particular predisposition. Even before the genes are cloned, linkages in mice can be related to the human genome by comparative mapping, providing markers to be used to survey populations of individuals who have experienced sepsis, ARDS or MODS. Such analyses could provide genetic markers that identify individuals with a specific predisposition for an exaggerated inflammatory response. Genes regulating the degree of inflammatory response in mice are candidates for human disease and may ultimately provide a basis for identifying individuals at risk for an exaggerated inflammatory condition.

One of the best known examples of the power of a mouse genetic approach to the study of inflammation is the C3H/HeJ mouse strain, which is hyporesponsive to *E. coli* LPS. This response to LPS was attributed to the presence of the *Lps*^{d} allele on mouse chromosome (Chr) 4, which was not present in other strains such as C3H/HeOuJ or C3H/HeN (82, 83). The defect in these C3H/HeJ mice was shown by positional cloning (the process of identifying a mutant or modifier gene based on its localization on the genetic and physical levels, followed by a candidate gene assessment) to arise from a mutation in *Toll-like receptor 4 (Tlr4)* (84). The *Toll* gene is involved in resistance to fungal infection in *Drosophila* (85). Human patients with *TLR4* mutations have a pattern of sensitivity to LPS and IL-1 similar to *Lps*^{d} mice (86). Today, it is known that *TLR4* is a central component of the response to injury and innate immunity and it is being studied extensively for its significant role in the extremely complex clinical picture of trauma. Clearly, the importance of *TLR4* greatly exceeds its role in the LPS response of one inbred mouse strain. This is a noteworthy example of the fundamental experimental strategy of working with limited components of a complex system to gain insights that can be extrapolated to the whole. The identification of candidate genes involved in the inflammatory response in a mammalian system is an important tool for gaining insight into the genetic pathways that mediate the inflammatory process. Cloning of genes that contribute to heritable strain differences will provide the information necessary to understand at a molecular level how these variants contribute to differential phenotypes. The role of these genes in more complex and clinically relevant animal models of injury can be studied. In addition, polymorphisms in particular genes can be translated into genetic markers that ultimately act as predictors for the clinical outcome of critically ill patients.

EXAMPLES OF MOUSE GENETIC APPROACHES TO UNDERSTANDING THE RESPONSE TO LPS

The quantitative differences in LPS response between B6 and A/J mice were used to map loci that contributed to LPS-induced inflammatory processes. Several mouse genetic resources were used to accomplish this objective. RI mouse strains have been generated by inbreeding the progeny (sisters and brothers) of two given mouse strains (e.g., B6 and A/J) more than 20 generations to produce new inbred strains homozygous for a unique recombination of the alleles that differ between the founders (87). Because RIs are inbred, they provide a genetically homogeneous population that can be assayed for a particular condition multiple times. Moreover, hundreds of genetic markers have been placed on these strains to provide a "pregenome scan." An example of the utility of RIs was demonstrated in the analysis of splenic B-cell proliferation in response to LPS. Splenocytes obtained from naïve B6 or A/J mice showed a different rate of proliferation after incubation with LPS in culture conditions. Through this paradigm, linkage to four loci on Chr 1, 7, 11, and 13 was obtained by mapping 26 different RIs derived from B6 and A/J mice (BXA, AXB). This analysis also demonstrated a weakness of RIs. The relatively small number of strains in each RI set meant that mapping results were generally of low statistical significance. We circumvented this problem by using congenic mouse strains to independently confirm two of the four loci (88). A congenic strain contains a particular locus of one strain; preexisting congenic and consomic strains provide a significant shortcut to high-resolution mapping for the identification of the involved genes by positional cloning (87).

Differences in the infiltration of PMN within the liver after LPS challenge were observed between B6 and A/J mice after a whole-body pathological analysis (22). The genes contributing to this phenotype were also mapped. The initial analysis using the AXB, BXA RI strains resulted in suggestive linkage to four loci. An intercross strategy (consisting of the crossing of the F_1 generation between B6 and A/J mice) was used to examine this trait further. Analysis by genome scanning of 124 intercross mice (representing 248 meioses) between B6 and A/J revealed a significant linkage to a locus on Chr 13 and confirmed suggestive mapping results from RI strains for a locus on Chr 5 (89). These two loci showed an epistatic relationship (i.e., a phenotypic interaction of nonallelic genes) such that the locus on Chr 5 had an impact on the PMN infiltration phenotype only if it was homozygous for the B6-type allele in the presence of a homozygous B6 phenotype at the locus on

Chr 13. This is the type of interaction that we would predict to account for the complex response to endotoxic or septic shock in human patients. This study illustrates the power of the intercross to map complex traits.

CONCLUDING REMARKS

Results from different laboratories clearly support our central thesis that the inflammatory process is modulated by the genetic background. In addition, we have demonstrated the feasibility of using mouse genetics to map loci involved in the inflammatory process, using unique combinations of mouse resources to demonstrate genetic contributions to the response. This approach will allow us to identify additional genes that modulate the inflammatory response by considering different aspects of the phenotype. Moreover, our observations have illustrated that a combination of different initiating insults and genetic backgrounds contribute to differing responses. Consequently, the incorporation of these factors into the experimental animal models used to study possible therapies may result in more adequate monitoring of the clinical situation and increase the chance of successful clinical trials. Additionally, defining genetic variation in the inflammatory response may lead to the identification of patients who are likely to have different responses to therapy. Thus, our efforts should be directed at introducing and standardizing the appropriate variables that modify the response to injury (i.e., genetics, sex, age and environment) in our experimental animal model to obtain information that could be applicable to the treatment of human beings.

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