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Commentary

Cerebral Malaria Pathogenesis

What Can We Learn from Microarray Analysis?

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Cerebral malaria (CM) attributable to *Plasmodium falciparum* infection is estimated to affect 575,000 children in sub-Saharan Africa every year[1] and is among the deadliest forms of malaria, with an average estimated mortality rate of 18.6%.[2] Although extensive studies have been conducted in murine models of CM and in human populations with CM, the pathogenesis of CM is still incompletely understood. Studies to date suggest that CM is attributable to a combination of local brain tissue damage from microvascular ischemia and hypoxia and more global brain injury caused by the host immune response to the parasite.[3] A deficiency of most murine and human CM studies is that assessment has been restricted to specific predefined factors hypothesized to be of importance. These studies address specific hypotheses but cannot provide a systemic evaluation of the potential factors in the pathogenesis of CM.

Systems biology, in particular the decoding of the human and murine genome, development of microarray analysis, and application of more sophisticated computational technology to assess the results of this analysis, has moved us ahead in our understanding of numerous diseases. In an elegant and carefully designed series of experiments in this issue of *The American Journal of Pathology*, Lovegrove and colleagues[4] use microarray analysis of whole brain tissue gene expression in CM-susceptible and CM-resistant mice to define potential pathways involved in murine CM pathogenesis. Their microarray analysis and confirmatory quantitative real-time polymerase chain reaction (PCR) and immunohistochemistry studies demonstrate that interferon (IFN)-regulated processes and neuronal apoptosis appear to be important in murine CM pathogenesis. These findings, particularly the findings about neuronal apoptosis, are novel and add significantly to our understanding of murine CM pathogenesis. Although there are important differences between murine CM models and human CM, these findings may also provide clues about human CM pathogenesis and, in particular, suggest potential mechanisms for the long-term cognitive sequelae that occur in children with CM.[5,6]

Differences between Murine CM Models and Human CM

As is the case for many diseases affecting the brain, far more is known about the pathogenesis of murine CM than human CM. The most obvious reason for this is that sizeable numbers of murine brains can be studied at different phases of the illness, but human brain studies of CM are limited to those done at autopsy. Another reason for the paucity of information on human CM pathogenesis is that human CM occurs almost exclusively in low- and middle-income countries. The resources in these countries for investigating CM pathogenesis are severely limited, and the resources provided by wealthier countries for such studies in malaria endemic areas have to date been relatively meager. Despite these limitations, in addition to cultural problems with acceptance of autopsies in children who die of CM, remarkable human brain autopsy studies have been conducted in malaria endemic areas, most notably the studies conducted in Malawi by Taylor and colleagues.[7] These studies have added significantly to our understanding of pathological processes that occur in human CM, but even in the best of these studies, the number of autopsy specimens studied has been fairly small. In addition, these studies have not been able to assess the changes that occur before death or the changes that are present in children who survive CM. For these reasons, murine studies have been an important source of additional insight into the pathogenesis of CM.

However, there are important differences between murine CM models and human CM. The most important difference is that *P. falciparum*, the *Plasmodium* species that causes CM in humans, is not infectious in mice, so murine

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malaria models generally use either the rodent malaria species *Plasmodium berghei* ANKA (PbA) or *Plasmodium yoelii*. A key difference between these parasites and *P. falciparum* is that *P. berghei* and *P. yoelii* infection in mice primarily induces adherence of leukocytes to vascular endothelium,[8] whereas *P. falciparum* infection in humans primarily induces adherence of infected red blood cells to vascular endothelium.[9] Murine models of CM are also not invariably associated with the human clinical picture of neurological impairment and coma. Using a combination of a specific *Plasmodium* species (PbA) and specific mouse strains (C57BL or CBA), researchers have developed fatal murine CM models in which the mice develop neurological impairment and coma, and sequestration of leukocytes and some red blood cells is seen.[10] However, 100% of mice in these fatal murine CM models die after contracting CM, whereas mortality rates in humans with CM are generally less than 40%. A resolving CM model has also been devised, in which DBA mice inoculated with PbA develop neurological symptoms, such as disturbed gait and transient limb paralysis, but recover from these symptoms. However, these mice never develop coma, the hallmark criterion for human CM.[8] The imperfect clinical approximation of the murine CM model, the difference in infecting *Plasmodium* species, and the differences between the murine and human immune system all suggest that caution must be exercised when attempting to extrapolate the results from murine CM models to human CM. Murine models can nonetheless provide us with a useful starting point for investigation of potential mechanisms of CM pathogenesis in humans.

What Can Microarray Analysis Tell Us about Murine CM Pathogenesis?

The hallmark of human CM pathology is the presence of infected red blood cells and leukocyte sequestration in the postcapillary venules of the brain.[9] The presence of local ischemia or hypoxia resulting from sequestration was long thought to be the cause of the symptoms of CM, but this would be expected to result in stroke-like events and does not satisfactorily explain the coma seen in CM or the often rapid recovery noted. Murine models provided early evidence that the host's immune response to the parasite played a critical role in the pathogenesis of murine CM. Throughout the past 2 decades there has been increasing evidence that a number of immune-related mechanisms first noted in murine CM, such as tumor necrosis factor (TNF)-α production,[11] blood-brain barrier breakdown,[12] and endothelial cell damage,[12] are also involved in the pathogenesis of human CM.[13–16] Human and murine data also suggest a potential role for other immunological or inflammatory factors in CM pathogenesis, including vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1), heme oxygenase-1, nitric oxide, kynurenic acid metabolites, and endothelin-1.[3,17] Current models of human CM postulate a contribution of multiple factors, including microvascular sequestration and blockage leading to local ischemia; cytopathic hypoxia, in which oxygen supply is adequate but cellular use of oxygen is not; and up-regulation of numerous immune or immune-related responses, all of which combine to lead to blood-brain-barrier breakdown, microglial and astrocyte activation, and damage or death of microglia, astrocytes, and neurons.[3]

The best studies done with microarray analysis provide new insights: as stated in a *Nature Immunology* editorial, there is nothing wrong in starting with a "fishing expedition" if the discoveries from that expedition are further characterized with nonmicroarray tools in a way that provides "fresh mechanistic insight into the immunological process being examined."[18] The methods of microarray analysis are as important as the microarray testing itself: appropriate and rigorous statistical analysis is key to accurate interpretation of results, and the methods and presentation of data analysis can make a big difference in communicating the main findings from within the many. In this regard, the inclusion of a gene-gene interaction network to create an "interactome" that identified hubs of interconnectivity is a major strength of the study by Lovegrove and colleagues,[4] allowing them to identify factors that appear to be particularly important in directing the overall transcriptional response to PbA infection. The finding that expression of several genes involved in regulation of IFN were up-regulated in CM-susceptible mice led to assessment of brain tissue by quantitative real-time reverse transcriptase (RT)-PCR directed at a number of immune-related genes. Expression microarray and PCR analyses correlated well for most genes, and the majority of up-regulated genes documented by RT-PCR were either involved in IFN signaling or were IFN-inducible.[4] In addition, the identification of several caspase genes and *Fas* (*CD95*) as prominent hubs in the interactome led the investigators to assess the brains of CM-susceptible and CM-resistant mice for evidence of apoptosis by terminal dUTP nick-end labeling immunohistochemistry. The expression microarray findings were again confirmed: CM-susceptible mice showed a higher number of terminal dUTP nick-end labeling-positive cells, primarily in the neurons of the cerebral cortex and cells of the leptomeninges.[4] The corroborative studies performed by Lovegrove and colleagues[4] add significantly to the data from initial microarray analysis and convincingly suggest a role for IFN-related processes and neuronal apoptosis in murine CM pathogenesis. Although other studies have suggested the importance of IFN, particularly IFN-γ, in murine CM,[19] only two very recent studies have documented neuronal apoptosis in murine CM.[20,21] Lovegrove and colleagues[4] confirm these findings of neuronal apoptosis in murine CM and also provide, through microarray analysis, a number of potential pathways by which neuronal apoptosis may occur. The study findings of Lovegrove and colleagues[4] nicely demonstrate the potential for microarray analysis to lead to significant new discoveries in murine CM pathogenesis.

Pitfalls of Microarray Analysis: Comparing Murine CM Studies

The report by Lovegrove and colleagues[4] is not the first to use microarray analysis to assess murine brain re-

sponses to CM, and indeed there are differences between this study and the previous studies that will require further investigations for resolution. The previously published studies of brain tissue gene expression microarray analysis in murine CM had slightly different foci than the study by Lovegrove and colleagues,[4] Sexton and colleagues[22] focused primarily on gene expression in splenic tissue, noting, however, that IFN-γ was present in brains of Balb/C mice at days 3 and 5 after infection and observing an increase in many IFN-inducible gene transcripts in the brain and splenic tissue of infected mice. In the study by Sexton and colleagues,[22] only C57BL/6 (CM-susceptible) mice were assessed; there was no comparison to CM-resistant mice. Delahaye and colleagues[23] tested brain tissue gene expression in two strains of CM-resistant mice (BALB/c and DBA/2) and two strains of CM-susceptible mice (C57BL/6 and CBA/J) to evaluate the contribution of strain differences to differences in gene expression. Mice in this study were tested only at day 6, after development of CM symptoms in the susceptible mice. The study identified 28 genes for which expression differed strongly between the susceptible and resistant mice, regardless of strain, but also identified several genes for which expression differed between the two different susceptible strains or the two different resistant strains.[23] The majority of genes that differed in level of expression between CM-susceptible and CM-resistant mice in the study by Delahaye and colleagues[23] were not "hub" genes in the interactome described by Lovegrove and colleagues,[4] but IFN-regulating genes were an important component of both gene expression sets. The findings of Lovegrove and colleagues[4] thus confirm the findings in the two previous murine CM microarray studies that IFN and IFN-regulating processes play a major role in murine CM pathogenesis. Interestingly, although earlier murine studies have implicated TNF-α in CM pathogenesis,[10] and TNF-related or TNF-induced genes were among the genes up-regulated in CM-susceptible but not CM-resistant mice in the study by Delahaye and colleagues,[23] TNF-α-related genes were not a major part of the interactome hubs in the study by Lovegrove and colleagues.[4] In contrast, neither any of the caspase genes nor *Fas* (*Cd95*) was noted to be up-regulated in the studies by Delahaye and colleagues[23] or Sexton and colleagues.[22]

The brain tissue microarray studies performed thus far in murine CM show a number of similarities and most strikingly point to IFN-regulating and IFN-regulated processes as critical to CM, but they also differ in their findings. There are a number of reasons why the findings may differ, including differing cDNA libraries used in the microarrays, differences across and within mouse strains, different methods and cutoffs for choosing statistical significance, and different analytic techniques. The similar findings provide a strong case for the importance of IFN-related processes in murine CM. The differences suggest a need for greater consistency in murine CM models and microarray statistical analyses and models if results are to be compared or considered generalizable. The differences also demonstrate the importance of corroborative data in these models, as evidence that up-regulation of gene expression does lead to the predicted outcomes.

Learning about Human CM from Murine CM Microarray Analysis

The differences in murine CM gene expression microarray studies indicate that further caution is required when attempting to extrapolate from murine findings, particularly from a single study, to human CM. The murine studies are best thought of as a launching pad for hypotheses and testing in human CM. For example, the study by Lovegrove and colleagues[4] again suggests a role for IFN-regulated or IFN-related processes, including IFN-γ production, in murine CM. Are type I and type II IFN expression important in humans with CM? The study by Lovegrove and colleagues[4] and two other recent studies[20,21] strongly suggest a role for neuronal apoptosis in murine CM. Could neuronal apoptosis be a cause of the neurological and, in particular, the long-term cognitive deficits seen in children with CM? The study by Lovegrove and colleagues[4] also demonstrates up-regulation of the gene regulating endothelin-1 production. Might endothelin-1 production leading to local vasospasm be part of the pathogenesis of human CM? Finally, earlier studies, but not the study by Lovegrove and colleagues,[4] suggest a role for TNF-α in murine CM. Is TNF-α truly a mediator of murine CM or a marker for other processes? Does TNF-α play a role in human CM?

Testing these hypotheses in humans will not be easy. Autopsies remain the only way of assessing brain tissue in humans with CM and present the insoluble problem of providing tissue only from those with terminal disease. Microarray analysis of brain tissue could provide important information but might primarily reflect terminal responses to CM, as opposed to the responses that occur in all children with CM, including survivors. Measuring certain factors, such as endothelin-1 or TNF-α, in cerebrospinal fluid might provide an indication of their production in the CNS,[24] although local production may be missed with these measurements. Assessment of vasospasm may be aided by imaging technologies such as magnetic resonance angiography,[25] but such technologies are rarely available in malaria endemic countries. Microarray analysis of gene expression in peripheral mononuclear cells could give a picture of the systemic response to *P. falciparum* infection that leads to CM, but gene expression in the brain may be very different from that in other tissues such as the liver or spleen, as Lovegrove and colleagues[26] have demonstrated in an earlier murine CM study. Future studies of human CM will require increased support of scientific and clinical infrastructure in malaria-endemic countries and increased support of research specifically assessing CM and its complications in these countries.

Attempts should be made to determine what putative factors can be assessed in human studies, whether directly, by brain tissue microarray analysis, immunohistochemistry, real-time RT-PCR, or other methods, or indirectly, by methods such as CSF analyte measurement.

There are enough differences between murine and human CM that going directly to human therapeutic trials from murine data seems premature, but murine CM studies may provide the basis for human studies that lead to therapeutic trials. Assessment for factors such as neuronal apoptosis in human CM may provide new insights into the pathogenesis of long-term morbidity from CM and lead to therapeutic trials to decrease not only mortality but also morbidity. Support for both human and murine studies of CM is clearly necessary if we are to advance our understanding of CM pathogenesis and move on to successful adjunctive therapy that will decrease CM morbidity and mortality. Thoughtfully designed and carefully performed gene expression microarray analyses like those in the report by Lovegrove and colleagues[4] in this issue tell us something new about murine CM pathogenesis and may well lead to a better understanding of human CM pathogenesis.

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