MS AND OTHER ANALYTICAL METHODS

BY THOMAS HAYDEN

Making Inroads on
Malaria

Standing on a pristine island beach just a couple dozen miles off the Pacific coast of Panama, Todd Capson should be a scientist in his element. But the biodiversity conservation specialist can't seem to focus on the vibrant coral reefs in front of him nor on the nearly unbroken swath of virgin rainforest that covers the island of Coiba behind him. He's a research associate of the Smithsonian

AID THE DETECTION AND UNDERSTANDING OF THE PERSISTENT PARASITE PLASMODIUM

Tropical Research Institute and a project leader with the U.S.-financed International Cooperative Biodiversity Groups program. But his mind is stuck firmly back in a Panama City lab, and on one of the darker sides of tropical paradise—malaria. The connection? A new bioassay for antimalarial agents, developed by his colleague Eduardo Ortega Barria of Florida State University's Panama-based Institute for Tropical Medicine and Health Sciences (*1*). "Tropical diseases like malaria are absolutely devastating," Capson says, "and there's a good chance that the cures might be right here" in the form of novel chemicals from the island's plants and marine life.

Capson is hoping that the biomedical potential of natural products can help ensure conservation of the tropical ecosystems where they are found. But the quest for new antimalarials reaches far beyond the trial-and-error approach of natural-products drug discovery. Since the 2002 genome sequencing of *Plasmodium falciparum*, the most deadly of the four species of parasite that cause malaria in humans, the molecular details of the infection process are finally being laid bare (*2*). In labs around the world, scientists are uncovering new information about the biology, genomics, and proteomics of malaria infection. They are developing improved diagnostic methods, identifying new drug-development targets, and searching for a malaria vaccine.

A parasitic disease with a complex life cycle (Figure 1), malaria is caused by protozoans of the genus *Plasmodium*, delivered by the bites of *Anopheles* spp. mosquitoes (previous page), resulting in ~500 million cases of malaria infection worldwide per year. Malaria is a serious problem throughout the tropical world, but its impact is particularly severe in impoverished areas. The social and economic effects of widespread infection are profound, and at least 1 million, and perhaps as many as 3 million, people die of the disease every year; ~90% of those deaths occur in Africa. The disease once ranged well into subtropical areas, but thanks to the aggressive mosquito-eradication projects of the 1950s, it was extirpated from large areas and largely controlled in others. Malaria deaths in India, for example, fell from a high of 800,000 per year to nearly zero. The disease is starting to rebound, however.

It is expanding its range again and developing resistance to many of the drugs once used to treat it. Those drugs include the cheapest options, such as chloroquine, which are often all that are available in impoverished areas.

For humans, malaria begins when an infected female *Anopheles* mosquito visits for a blood meal. Inadvertently, she injects *Plasmodium* sporozoites—the sexually produced infective stage of the parasite—that have taken up residence in her salivary glands. Within an hour, the sporozoites make their way through the bloodstream to invade liver cells, where they reproduce asexually to generate thousands of merozoites. At this stage of its life, the parasite invades red blood cells and hijacks cell machinery to aid its own reproduction, which can occur repeatedly in cycles lasting ~24 h. Each time, the invaders rupture their host blood cells and
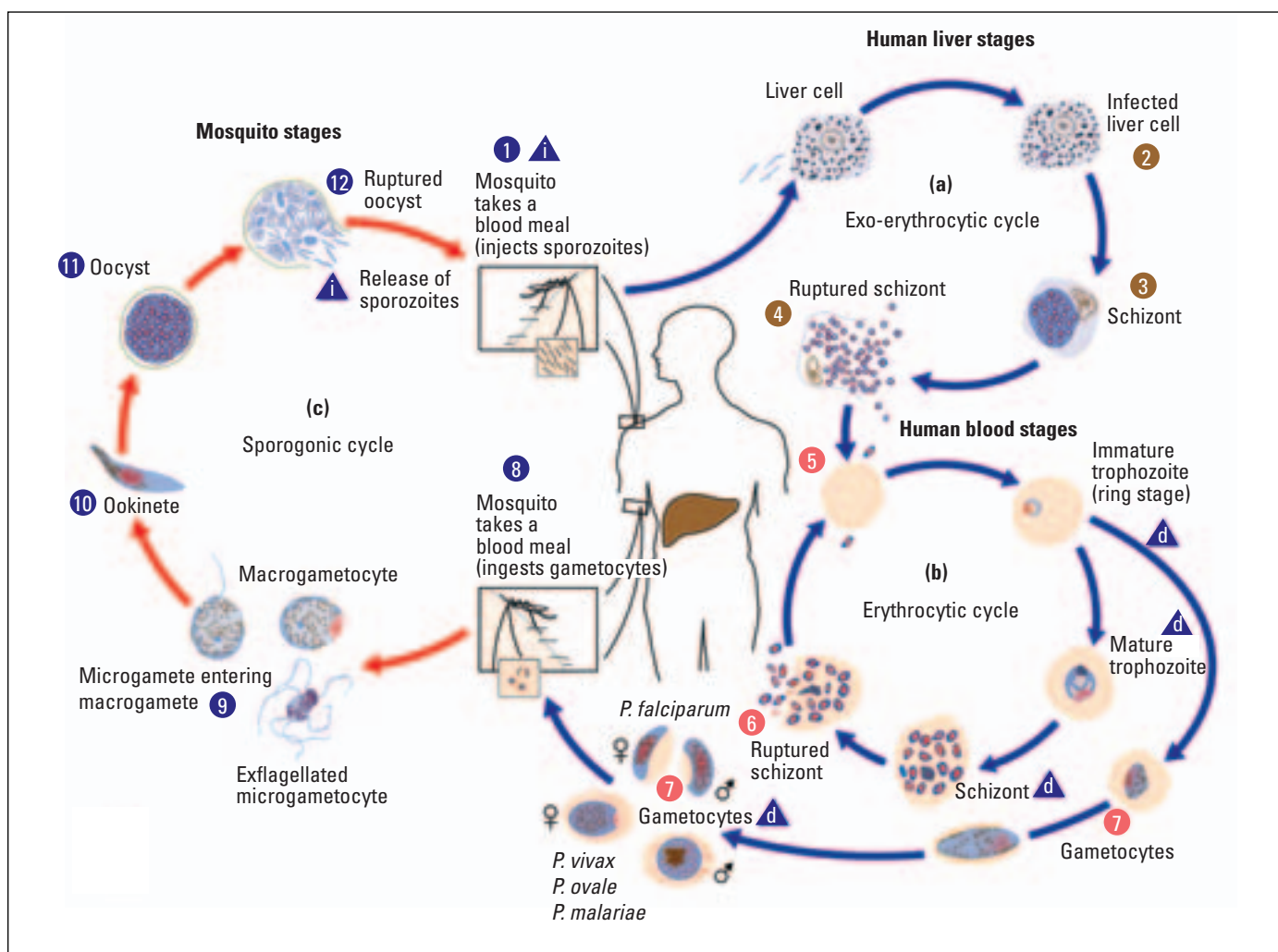

FIGURE 1. The life cycle of the *Plasmodium* parasites.

A female mosquito transfers sporozoites to a person as she draws blood (stage 1). (a) Sporozoites infect liver cells (stage 2) and enter the exo-erythrocytic replication cycle. The sporozoites mature into schizonts (stage 3), which rupture and release merozoites (stage 4). Note that *P. vivax* and *P. ovale* can persist in a dormant state, called hypnozoites, in the liver and cause relapses of the disease weeks, months, or years later. (b) The parasites multiply asexually in red blood cells; this is the stage at which the human host has clinical symptoms of disease. Merozoites infect the red blood cells (stage 5), and ring-stage trophozoites mature into schizonts, which rupture and release merozoites (stage 6). Some parasites differentiate into gametocytes for sexual reproduction (stage 7). As a mosquito draws blood from an infected human, she ingests male and female gametocytes (stage 8). (c) The parasites reproduce within the mosquito during the sporogonic cycle. Zygotes are produced in the mosquito's stomach (stage 9). They become motile and elongated (stage 10) and invade the wall of the midgut, where they become oocysts (stage 11). The oocysts grow, rupture, and release sporozoites (stage 12), which migrate to the mosquito's salivary glands. i: infective stage; d: diagnostic stage.
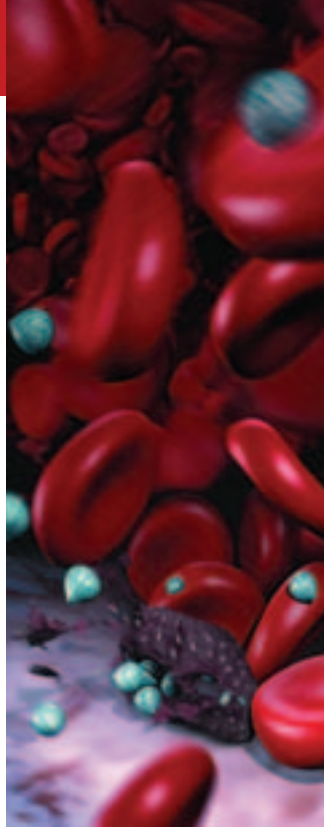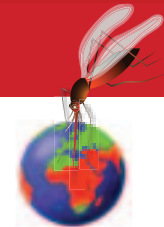
Sticky cells.

Red blood cells burst and release the parasite in merozoite form. During the stage when red blood cells are infected, the human host feels ill.

cause the disease's characteristic fever, chills, and anemia. Over time, some of the merozoites develop into male and female gametocytes, which can infect new mosquitoes as the insects feed. They develop into gametes in the mosquito's gut and complete their life cycle there by fusing together to form an oocyst—the source of a new round of sporozoites.

Standard diagnosis of malaria has been by microscopic detection of the parasite in blood smears (Figures 2 and 3); it's been that way since soon after *Plasmodium* was identified in 1880. The tried-and-true approach has several advantages, notes Patrick Duffy, director of the Malaria Antigen Discovery Program at the Seattle Biomedical Research Institute. In particular, it can determine the number and species of parasite— key parameters for deciding on appropriate treatments. But microscopy has limitations as well. Microscopes or electricity may not be available in many of the impoverished areas where malaria is a serious problem, and training and expertise are needed to make accurate diagnoses. Kathy Mangold, a research scientist at the Evanston Northwestern Healthcare system in suburban Chicago, adds that skilled pathologists in developed nations typically don't see malaria specimens often enough to maintain their expertise. And the price of expert microscopy, which can take an hour or more per patient, can be prohibitive even in wealthy countries. "The sample preparation doesn't cost much," says Mangold, "but the cost of experience is very high."

The price of misdiagnosis, or of no diagnosis at all, can be equally high. In the developed world, clinicians may not recognize the symptoms exhibited by travelers returning from malaria-infested areas; this can delay treatment. And wrong or missing information on the parasite's species or drug resistance can further endanger the patient's health. This is especially true in the case of *P. falciparum*, which causes just 50% of malaria infections but is responsible for 90% of the deaths. In developing regions, Duffy points out, clinicians may assume that malaria is behind any fever they encounter. This can lead to overuse of antimalarials, which may contribute to the development of drug resistance. It also may cause increased mortality of febrile patients who are falsely diagnosed with malaria and, therefore, not treated for their actual illnesses.

According to Duffy, several emerging diagnostic techniques show promise in alleviating these concerns. These include immunochromatographic tests (ICTs) or dipsticks, PCR-based diagnostics, and laser desorption MS (LDMS) detection of the heme groups characteristic of *Plasmodium*-infected red blood cells. The primary benefits of ICTs include rapid diagnosis—typically ≤20 min—an essential attribute with acute febrile illness, and relative ease of use, which can be critical in low-resource environments. ICT dipsticks for malaria are commercially available and have shown good sensitivity and specificity for malaria diagnosis compared with standard microscopy. In one study, conducted at a hospital in Thailand, the ICT-based NOW Malaria Test (from Binax, Inc., in Portland, Maine)—a test designed to differentiate between *P. falciparum* and the less lethal *P. vivax*— showed 100% sensitivity for *P. falciparum* and 87.3% sensitivity for *P. vivax*. The specificity for each species was also >96%, and when patients diagnosed with *P. malariae* were taken into account, the NOW ICT achieved 100% specificity for non-*falciparum* malaria (*3*).

But for all their advantages, dipsticks so far can only determine the presence or absence of *Plasmodium*. For example, they cannot provide any information on the number of parasites—an important parameter for ensuring the success of treatments. The detection limit of ICT dipsticks is >100 parasites/µL blood, compared with ~50 parasites/µL for a skilled microscopist. And although the ICT assays can cost as little as $1 per dipstick, Duffy points out that a full course of treatment with traditional drugs, such as chloroquine or pyrimethamine-sulfadoxine, can cost an order of magnitude less. Therefore, in cash-strapped economies, government officials may opt to simply treat all febrile illness with cheap antimalarials, rather than paying more for an accurate diagnosis. Duffy suggests that as more expensive combination therapies become routine first-line treatments for drug-resistant strains of malaria, that economic equation will start to change.
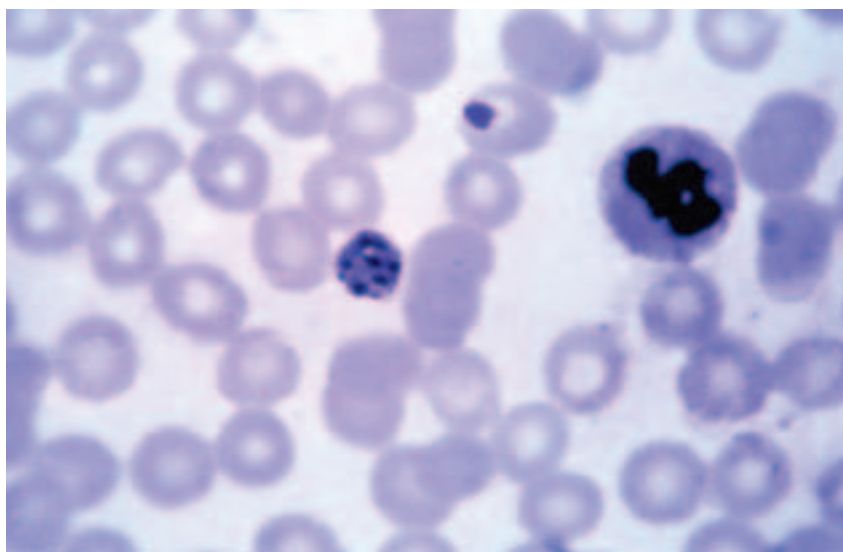
FIGURE 2. The culprit.

The gold-standard method for diagnosing malaria is to observe parasites within red blood cells. In this case, Giemsa staining reveals *P. malariae* schizonts.
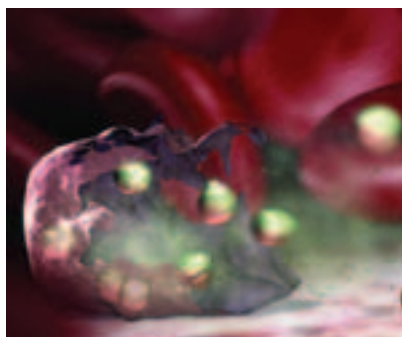
Beyond ICTs

PCR-based malaria diagnostics offer considerable improvements in sensitivity over both microscopy and ICT, typically resulting in a detection limit of <5 parasites/µL. The cost of PCR equipment and reagents renders the technique impractical for many areas in which malaria is endemic. However, the high sensitivity and specificity and the low requirement for experience and expertise make PCR an ideal diagnostic in pathology labs in developed countries. A team including Mangold and Richard Thomson, a microbiologist at Evanston Northwestern Healthcare, recently developed a real-time PCR assay, which the hospital system began using in early 2005. It has a detection limit of just 1 parasite/µL (4).

The Chicago suburbs may seem like an unlikely site for malaria research, Mangold admits, but in this three-hospital system, returning travelers account for several dozen requests for malaria testing per year. That's certainly enough to require diagnostic ability but not necessarily enough for the system's microscopists to maintain their expertise. And because of the malaria parasite's infectious cycles, blood microscopy can miss the infection altogether if blood samples are drawn during the wrong stage of the cycle. "Real-time PCR picks it up even at the times when the parasite isn't there in the blood," says Mangold. "The DNA is always there during an infection, even when the parasite is not visible microscopically, and may linger for days after all the organisms are eliminated from the bloodstream." The test targets 18s ribosomal RNA, Mangold says, and a real-time PCR system with melting-curve analysis can provide an accurate, sensitive diagnosis in <1 h.

Another benefit, says Mangold, is the high degree of species determination. "The distribution of different *Plasmodium* strains is somewhat geographic," she says, "but you can never depend on that." Mangold says that her hospitals, located near the major travel hub of Chicago, run several dozen malaria tests per year. (Every year, ~1500 cases are reported throughout the U.S., and ~60% of those occur in travelers who have returned from abroad.) Thick blood smears are used to determine the parasite load and thin smears for morphology, she says. "Then, we use the real-time PCR to confirm the diagnosis fast, while we have the febrile patient in the bed." The alternative, she says, would be the much lengthier processes of having slides confirmed at another institution. The PCR test also can be helpful in cases in which a patient is infected with more than one species of *Plasmodium* at the same time. "It's happened a couple of times," says Mangold. "The microscopist saw *falciparum* and missed *vivax*."

LDMS has recently become another promising option for malaria diagnosis. An LDMS system developed at the Johns Hopkins University, using direct UV LD TOFMS, can distinguish the mass spectral signature of heme derived from the break-down product hemozoin from that of hemoglobin-derived heme (5). This allows for detection of parasite-infected red blood cells, with the potential for a detection limit of ≤10 parasites/µL—but with no potential for parasite speciation. Duffy, who reviewed emerging malaria diagnostics in the *American Journal of Tropical Medicine and Hygiene* in 2005 (6), says that the technique shows promise but that its development is impeded by the intricate parasite life cycle. Quantification presents a particular problem, he says, noting that the parasite produces varying levels of hemozoin depending on its life-cycle stage. In particular, *P. falciparum* and the other species differ in which stages are present in peripheral blood circulation, further complicating quantification in cases of multispecies infection. Still, says Duffy, given the technique's potential for speed, ease of use, and relatively inexpensive operation (after the initial costs of purchasing the equipment), the procedure could be used for rapid screening of blood samples, followed by microscopy or dipsticks for speciation.

Another new detection protocol, based on a small-volume electrochemical enzyme-linked immobilized DNA hybridization assay for a *P. falciparum*-specific gene segment, is under development at the Arkansas-based biotech company Vegrandis (7). Targeting the *P. falciparum* CSP gene, the test is conducted in 50-µm-diam microwells that have self-contained microelectrodes. The technique has particular promise for use in drug discovery and vaccine development, according to Zoraida Aguilar of Vegrandis, because of its extremely low detection limit of 1.4 ng/mL target DNA; this limit corresponds to considerably fewer
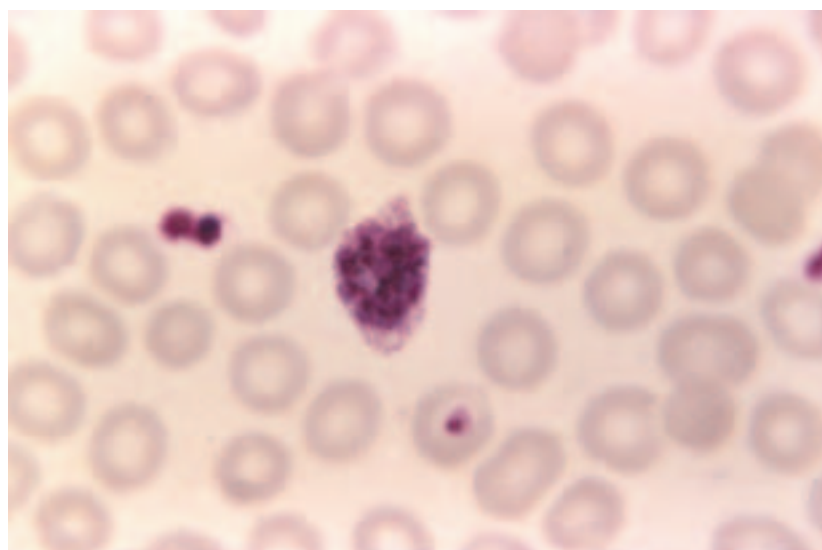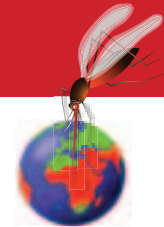
FIGURE 3. Signs of trouble.

P. ovale trophozoites are visible in this Giemsa-stained micrograph. Trophozoites mature inside red blood cells and mature into schizonts, which segment into merozoites. When the cell ruptures, some merozoites will repeat this cycle, whereas others will differentiate into gametocytes.

than 3–4 parasites/µL. That may actually be more sensitivity than is useful for clinical applications, according to Duffy. He cites evidence that submicroscopic malaria parasitemia in pregnant women, for example, has none of the issues, such as low birth weight and maternal anemia, presented by parasite loads that can be detected by microscopy. Aguilar points out the system's potential for automation, rapid diagnosis, and freedom from nonspecific interference when tested in human hepatocytes, pig liver, and chicken serum. She suggests that it could therefore become an important part of following parasite reaction in drug-delivery and vaccine-development studies in cell culture, as well as being useful for screening at blood banks and diagnosis in clinics.

Aguilar adds that the company recently received a grant from the National Science Foundation to develop a microarray-based detection system for the various species that cause malaria. The new microarrays will differentiate between *P. falciparum* and *P. vivax* by combining a genetic assay with an immunoassay, she says. Other species, such as *P. ovale* and *P. malariae*, will be incorporated as species-specific antibodies become available for these organisms.

The mosquito vacuum.

This 1965 photograph shows a truck-mounted mosquito vacuum, which collected mosquitoes from residential areas.

CDC/H. E. STARK

Toward new targets

The complexity of *Plasmodium*'s life cycle and its highly specialized mode of life are reflected in the parasite's unusual genomic makeup and protein expression patterns. This situation presents opportunities for the identification of unique molecular targets for drug and vaccine development, but it also creates considerable difficulties for researchers working with the complicated, unfamiliar system. The 23-Mb *P. falciparum* genome, which was announced in October 2002, consists of ~5300 genes on 14 chromosomes, including genes that allow the parasite to avoid detection by the human immune system and metabolic genes that lack human counterparts (*2*). All of these genes are potential targets for drug development. (The genome of *P. falciparum*'s primary mosquito vector, *Anopheles gambiae*, was announced simultaneously and contains ~14,000 genes [*8*]. In one recent LC/MS/MS proteomic analysis of salivary glands from female *An. gambiae*, researchers found a large proportion of novel proteins, which may represent potential targets for controlling pathogen development and/or transmission from the vector [*9*].)

Not surprisingly for a parasitic protozoan, the *P. falciparum* genome carries fewer genes for enzymes and transporters than do the genomes of other single-celled eukaryotes. A large pro-

portion of *P. falciparum* genes are involved in evading the human immune system and otherwise mediating the host–parasite interaction. It is the most (A + T)-rich genome yet sequenced—a factor that complicates cloning, sequencing, and assembly efforts—with an overall (A + T) composition of 80.6%. The introns and intergenic regions reach an (A + T) composition of 90%. And of the 5268 proteins predicted at the time of sequencing, ~60% lacked the similarity to known proteins necessary to assign probable functions. This very high proportion of apparently unique genes could be exacerbated by the extreme (A + T)-richness, the authors of the initial sequencing paper concluded, and it is also a clear indication of considerable evolutionary distance between *P. falciparum* and other eukaryotes that have been sequenced to date.

The wealth of genomic information now available for the malaria parasite and its mosquito vector is not easily translated into an improved understanding of the basic biology of the *Plasmodium* life cycle. Nor do practical applications, such as novel molecular targets for drug and vaccine development, emerge easily from the reams of sequence information. This is partly due to the inherent complexity of the disease and of the *Plasmodium* life cycle and partly a result of the parasite's unusual genome.

One recent effort to overcome these obstacles leverages protein–protein interactions to assign putative functions to some of the "hypothetical" genes that currently make up ~65% of the *P. falciparum* genome (*10*). "Most of the *P. falciparum* genes do not have enough homology to known genes to predict their functions," says Douglas LaCount of Purdue University, lead author on the paper. "The interactions are most directly useful when a

hypothetical protein is found to bind to a protein of known function. In that case, you can predict that the hypothetical protein is involved in the same process as the known protein."

These interactions, indications of proteins that form complexes together in vivo, were mapped for one-quarter of the proteins of *P. falciparum*, including novel proteins from each of the parasite's major bloodstream life-cycle stages. (Because libraries were derived from infected red blood cells, the analysis did not include proteins expressed exclusively during the liver-infection or mosquito phases.) The team of researchers, led by Stanley Fields of the Howard Hughes Medical Institute and the University of Washington and by Robert E. Hughes, now at the Buck Institute in Novato, Calif., identified 2846 unique protein interactions, the majority of which included at least one previously unknown protein.
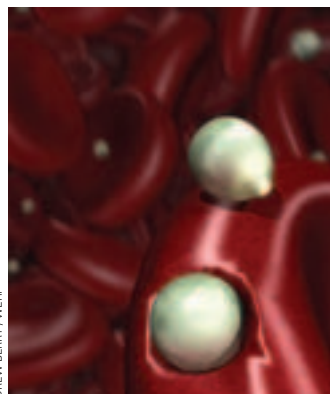
The method, based on the yeast two-hybrid assay, avoids the typical problems of expressing the parasite's genes in *Saccharomyces cerevisiae*. "The base composition of the genes of *Plasmodium* is very different from that of yeast or bacteria," says another member of the research team, Marissa Vignali of the University of Washington. Because of the protozoan's elevated (A + T) content, "It is very difficult to express *Plasmodium* proteins in these species." Instead, the researchers fused random *P. falciparum* protein segments with the Gal4 DNA-binding or activation domain and with an enzyme. The enzyme allowed for growth of auxotrophic transformed yeast in such a way that only yeast transformants containing in-frame and expressed *Plasmodium* protein fragments would grow. Libraries of DNA-binding-domain fusions, or baits, were generated and used to screen individual transformants against a library of activation-domain fusions, or preys. All told, the researchers say they identified >2000 non-overlapping gene fragments, representing ~1295 *P. falciparum* genes from the parasite's red blood cell life-cycle stages. "The number of putative interactions between *P. falciparum* proteins identified by this approach is impressive," Fields says. "But large protein interaction data sets generated by high-throughput yeast two-hybrid methods inherently contain a fraction of false positives, so some of the reported interactions will not prove to be biologically relevant."

The researchers surveyed the resulting protein interaction network, says Vignali, "seeking to identify clusters of proteins of particular interest for the understanding of the basic biology and pathogenicity of the parasite." One approach, she says, was to look for clusters of higher-than-expected interconnectivity, an indication of functionally related proteins. A total of 96 subnetworks showed elevated connectivity. The most highly connected cluster of proteins in the *P. falciparum* interconnectivity network appears to be involved in processes such as transcriptional regulation, mRNA stability, ubiquitination, and integration of chromatin modifications. The completed *Plasmodium* genome sequence revealed several apparently unique gene expression characteristics, LaCount says, yet encoded few recognizable transcription factors. "We think that this set of interactions is somehow involved in regulating gene expression," he says. "We don't really know much about how gene expression is regulated, but one hypothesis is that the proteins in the cluster with DNA binding domains bind to particular locations in the genome and recruit other members of the complex that then affect chromatin structure or transcription."

A second cluster of highly connected proteins is apparently involved directly in host-cell invasion. The cluster includes a leading vaccine target candidate, merozoite surface protein 1 (MSP1). Though the specific role of MSP1 is unknown, an MSP1-coated cell surface is thought to be an essential element allowing merozoites to invade the host's red blood cells. By following temporal patterns of gene expression through the merozoite production cycle, LaCount and his co-workers identified a large network of co-expressed proteins, with expression peaks at the time of merozoite formation. The core of that subnetwork, they found, links 19 uncharacterized proteins to 16 others known to be localized on the merozoite surface or to be involved in the invasion of host cells. Interestingly, some of the proteins in the subnetwork localized either to the apical region of the developing merozoites or to rhoptries, secretory organelles typical of apicomplexan protozoa, which include *Plasmodium* and the causative agents of the diseases cryptosporidiosis and toxoplasmosis. "The basic knowledge we generated will now allow malaria researchers to formulate testable hypotheses about the function of unknown proteins of the parasite," says Vignali.

Some of that work has already begun. Silpa Suthram, Taylor Sittler, and Trey Ideker of the University of California, San Diego, published a companion paper alongside the primary data, consisting of a comparative study of subsets of the *Plasmodium* protein network (*11*). Their findings—that the network diverges significantly from those of other eukaryotes—are perhaps not terribly surprising, given the parasite's unusual mode of living. But the specific differences identified by these researchers may well prove to include important candidates for selective targeting of the malaria parasite.

In comparisons with the protein interaction networks of *S. cerevisiae*, *C. elegans*, *Drosophila melanogaster*, and *Helicobacter pylori*, *P. falciparum* showed just three complexes conserved with *S. cerevisiae* and none with any of the other model organisms. (Yeast and fly, by comparison, had the highest degree of conservation, with 61 conserved complexes.) This lack of interologs—subsets of the original interaction networks that are conserved across species—may have resulted in part from sampling issues. For example, the primary data on the *P. falciparum* network were derived largely from the asexual stages of the parasite's life cycle. But Suthram et al. concluded that a large degree of the uniqueness is due to true functional differences necessitated by *P. falciparum*'s parasitic lifestyle.

One of the yeast interologs, localized to the cell membrane at sites of polarized growth, is apparently involved in calmodulin-mediated endocytosis. The result is particularly relevant given
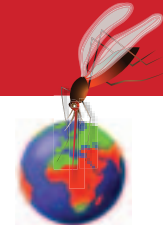
that calmodulin inhibitors have been demonstrated to reduce growth rates and suppress chloroquine extrusion—a key mechanism of resistance to the drug—in malaria parasites. In addition, endocytosis effects recently have been linked to the mechanism of both chloroquine and artemisinin, another plant-derived antimalarial that shows promise in combating chloroquine-resistant disease. Among the nonconserved networks, Suthram et al. found many membrane-associated proteins. They think these proteins represent unique *P. falciparum* cellular machinery and, thus, potential targets for drug discovery.

So many complexities

The search for potential drug and vaccine targets in *Plasmodium* is significantly complicated by the existence of four individual malaria-causing species. Each has its own complex life cycle, specialized organelles, and other subcellular domains, all of which can play a part in establishing the disease. Protein expression levels change throughout the *Plasmodium* life cycle; thus, potential antimalarial drugs may exhibit significantly different efficacy depending on when they are administered. The parasites have evolved to evade detection by both invertebrate and vertebrate immune systems, so they exhibit considerable antigenic variability as well. As a result, many potential targets are slippery at best. DNA microarrays have been useful for the study of differential gene expression throughout the blood stages of malaria infection, when the organism is abundant and can be cultured in vitro. But those assays require relatively large samples of RNA (in the microgram range), making them of little use for studying gene expression in other key stages of the *Plasmodium* life cycle.

One approach is to use multidimensional protein identification technology (known as MudPIT), a combination of in-line, high-resolution LC and tandem MS. In one large study published in 2002, Florens et al. compared protein expression in *P. falciparum* sporozoites, merozoites, trophozoites (the form that multiplies within red blood cells), and gametocytes (*12*). Notably, they found that the proteome of the sporozoite—the stage that is transmitted from mosquito to human and is responsible for the initial onset of infection—differs significantly from that of the other stages. Nearly half of the proteins expressed in this infective stage were unique to sporozoites. Out of a total of 2415 proteins, only 152, most of them for housekeeping, were expressed in every stage studied. The researchers also found that many *var* and *rif* genes, the products of which form antigenically variant proteins on the surface of infected red blood cells, were also expressed in sporozoites.

The proteome of each stage showed its own unique characteristics. In merozoites, which are released from infected red blood cells into the bloodstream and then must adhere to and reinvade new erythrocytes, cell-surface proteins and proteins in the apical organelles take on a central importance for survival. Invasion is an active process that uses an actin–myosin motor, and the researchers found that component proteins, including merozoite cap protein-1, actin, myosin A, and myosin A tail domain interacting protein, were all abundant during the merozoite stage. Also plentiful were MSP1 and MSP2, which have been linked to immune-system evasion.

The trophozoite stage, which occurs entirely within red blood cells, lasts ~48 h. The initial stage, which typically lasts ~30 h, involves considerable modifications to the host cell; these changes

Scope of the problem.

Malaria is so persistent that symptoms of the disease were described in Chinese medical writings in 2700 B.C. These days, it is most common in tropical and subtropical areas, especially sub-Saharan Africa.

enable the parasite to transport molecules into and out of the cell, to modify the cytoadherence properties of the host cell, and to digest hemoglobin and other cytoplasmic contents. Accordingly, the proteomic analysis revealed proteins implicated in hemoglobin catabolism, including falcilysin and members of the plasmepsin and falcipain families. The final 18 h of the trophozoite stage involve nuclear division, merozoite formation, and release; several proteases not involved in hemoglobin catalysis are suspected of involvement in parasite release and/or the invasion of new cells.

The gametocytes are found in a male:female ratio of ~1:4, and cell structure differs significantly with sex. The male gametocytes are terminally differentiated and largely lack ribosomes, whereas female cells contain the ribosomes and other cellular machinery needed to kick-start translation upon fertilization. Proteomic analysis found high levels of gametocyte-specific transcription factors, RNA-binding proteins, and mRNA-processing regulator proteins. Mature gametocytes spend time arrested in the G0 cell-cycle stage. They were found to contain the components of the cell-cycle regulatory cascades needed to respond almost immediately to the signals that trip gametogenesis, namely xanthurenic

acid and a drop in temperature. The asexual blood stages of *Plasmodium* derive energy from glycolysis and pyruvate-to-lactate conversion, but the researchers found evidence of oxidative phosphorylation and mitochondrial tricarboxylic acid cycle enzymes in both gametocyte and sporozoite stages. In the case of the gametocytes, the existence of mitochondria that are already functioning seems to anticipate life in the mosquito vector, where they will be needed. It also helps explain why these stages are susceptible to antimalarials that target respiration, such as primaquine and the artemisinin-derived products.

Because sporozoites are an invasive stage, they must survive in the open bloodstream for up to several minutes after being injected by their mosquito vector. Immune-system evasion poses a special challenge for this stage, which is nonreplicative. Thanks to their cycles of asexual reproduction, the intraerythrocytic stages of *Plasmodium* are able to use antigenic switching of the variant antigen genes and evade the host's immune system. Because the sporozoites don't replicate, they can't deploy this strategy, but proteomic analysis revealed peptides derived from a large variety of *var* and *rif* genes, most of which were unique to the sporozoite stage. The expression of such a wide range of *var* and *rif* genes may point to the sporozoite strategy for immune-system evasion, Florens et al. concluded.

Sporozoites were also found to contain the full complement of apical complex proteins needed for successful invasion of host liver cells, including proteins associated with rhoptries, micronemes, and dense granules. Many of the proteins found in apical organelles, including micronemes and rhoptries, have a single transmembrane domain and are generally also found in the merozoite stage. Rhoptries, in particular, are thought to be involved in modifications of the surface of host cells, and thus their proteins are among the most promising malaria vaccine candidates. In one recent study of malaria patients in Kenya, the presence of antibodies to a rhoptry-associated membrane antigen, RAMA-pr, was found to correlate with increased resistance to *P. falciparum* infection (*13*).

The function of another organelle found in all malaria parasites, the apicoplast, is less clear, though it is essential for their survival. The plastid is homologous to the chloroplasts of plant cells and is involved in anabolic synthesis of fatty acids, isoprenoids, and heme, which may be exported from the apicoplast. It contains a limited genome, coding for just 30 proteins, with another 550 or so nuclear genome proteins thought to be targeted to the organelles. The function of most has not yet been determined, but because several metabolic and transport pathways in the apicoplast differ from host pathways, they offer another promising target for antimalarial development.
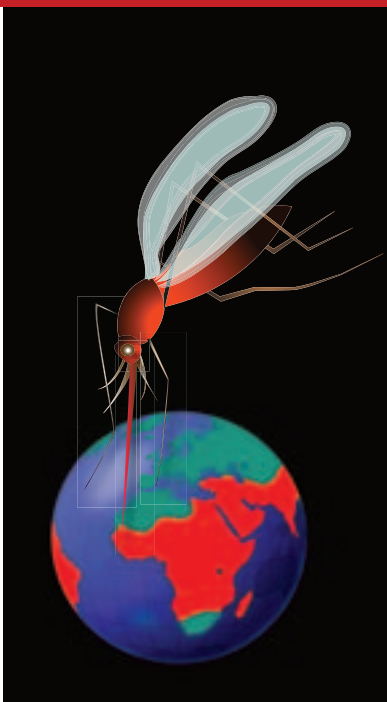
A full course of treatment with standard antimalarial drugs can be cheaper than diagnosing the fever.

A minor inconvenience?

Malaria treatment and prevention research has suffered from a lack of funding and research effort for years. But recently, renewed interest from both funders and the research community offers hope that a new generation of drugs and vaccines to counter the devastating effects of this complex disease may be just over the horizon. (In addition, public–private partnerships make large-scale projects such as the protein-interaction map possible, LaCount adds.) Proteomic analysis, improvements in bio-informatics, and the inrush of new information from genomic sequencing all lead to the hope that targets for drug and vaccine development can be identified. Is it possible that malaria might one day become a minor inconvenience, rather than a major factor in region-wide human misery?

In the meantime, diagnosis remains a most pressing issue—and we still have a long way to go, says Duffy. "Outside the realm of detecting malaria parasites is predicting disease," he says. "There are 500 million malaria infections each year but [just] 1 million deaths. Most infections will resolve without the requirement for treatment, while other infections are rapidly fatal." Clinicians lack the tools to predict whether patients will recover on their own or face an uphill battle with severe disease, he says. So, "while having cheaper, easier, and better tools to detect, speciate, and quantify parasites would be extremely valuable, it would be separately valuable for us to identify markers that predict poor outcomes and use these to develop diagnostic tools as well."

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