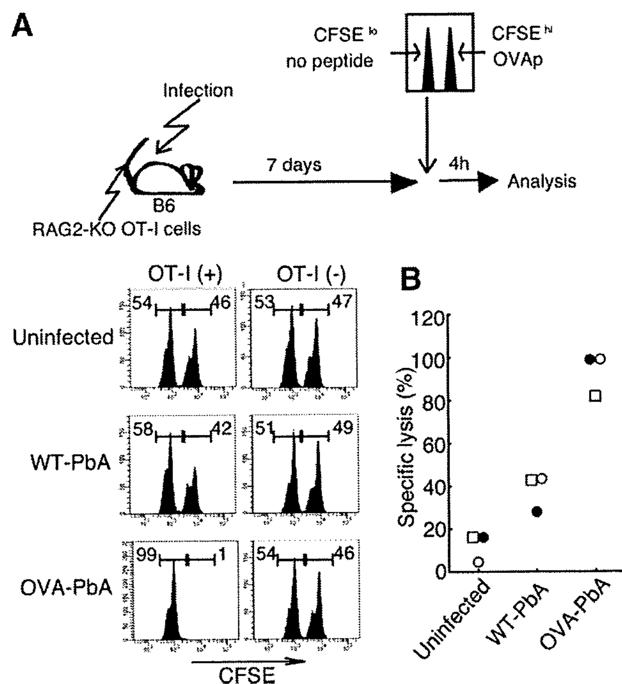

FIGURE 8. Cytotoxicity in vivo of OT-I $CD8^+$ T cells during PbA infection. *A*, B6 mice were uninfected or infected with WT-PbA or OVA-PbA and were inoculated with lymphocytes from RAG2-KO OT-I mice. Seven days later, mice received a 1:1 mixture of differentially CFSE-labeled target splenocytes (1×10^7), and the cytotoxicity was determined 4 h after target cell transfer. Numbers in plots represent the ratio of peptide-pulsed or unpulsed cells. Proportions of OT-I cells in $CD8^+$ T cells: uninfected, 2.7%; WT-PbA, 5.0%; OVA-PbA, 30.7%. Levels of parasitemia: WT-PbA (1.6%) or OVA-PbA (9.7%)-infected B6 mice with OT-I; WT-PbA (8.7%) or OVA-PbA (10.5%)-infected B6 mice without OT-I. *B*, Summary of percent-specific lysis of three similar in vivo cytotoxicity experiments. Result of the experiment in *A* (●) and two other similar experiments using B6 mice transferred with OT-I $CD8^+$ T cells (□, ○) are shown. The variation among experiments was not significant in each group of mice ($p = 0.97$, ANOVA for two-way layout data); hence the data in each group of mice were pooled to compare the difference in percent specific lysis; a significant difference was observed in both overall and paired comparison ($p < 0.05$, ANOVA for one-way layout data).

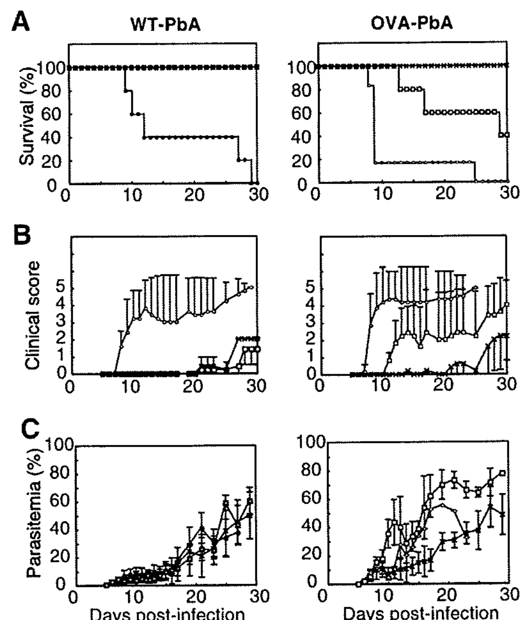

FIGURE 9. Involvement of $CD8^+$ T cell activation in the pathogenesis of PbA. B6 (○), RAG-2 KO (×), and RAG-2 KO OT-I (□) mice were infected with WT-PbA (*left*) or OVA-PbA (*right*). *A*, Kaplan-Meier estimation of survival distributions of mice infected with WT- or OVA-PbA. In WT-PbA-infected mice, survival time in B6 mice was significantly shorter than in other two groups ($p = 0.0018$, log-rank test). In OVA-PbA-infected mice, survival time in B6 mice was significantly shorter than in RAG-2KO OT-I ($p = 0.0165$) and in RAG-2 KO ($p = 0.0014$), whereas the difference in survival time was not significant between RAG-2KO OT-I and RAG-2 KO ($p = 0.0494$). Clinical scores (*B*) and levels of parasitemia (*C*) were also determined in each group after infection. Representative data of two similar results are shown.

To examine whether nonspecific activation occurs in $CD8^+$ T cells expressing other TCR during malaria infection, we used $CD8^+$ T cells from P14-transgenic mice, which express TCR specific for LCMV (26). P14 $CD8^+$ T cells up-regulated CD69, down-regulated CD62L, and expressed granzyme B after infection with WT-PbA, indicating that activation of $CD8^+$ T cells during PbA infection is not limited to OT-I cells (Fig. 6).

CTL function of T cells activated by malaria infection

To determine whether OT-I $CD8^+$ T cells that are activated during PbA infection are able to kill targets, we performed CTL assays in vitro. B6 mice were transferred with OT-I $CD8^+$ T cells and were infected with WT-PbA or OVA-PbA. $CD8^+$ T cells were enriched from these mice and were subjected to ^{51}Cr release assay (Fig. 7*A*). OT-I $CD8^+$ T cells in OVA-PbA-infected B6 mice showed specific CTL activity against OVA-pulsed targets. OT-I $CD8^+$ T cells from WT-PbA-infected mice showed weak but significant OVA-specific killing activity. We also examined CTL activity of $CD8^+$ T cells in RAG2-KO OT-I mice. These cells showed OVA-specific CTL activity after infection with WT-PbA or OVA-PbA, indicating that CTL can be induced without help of $CD4^+$ or other $CD8^+$ T cells (Fig. 7*B*). The CTL activity of OT-I $CD8^+$ T cells from OVA-PbA-infected mice was much higher than those from WT-PbA-infected mice, consistent with their higher expression of granzyme B (Fig. 5*B*).

We also evaluated in vivo killing activity of $CD8^+$ T cells during malaria infection. B6 mice were transferred or not transferred with OT-I $CD8^+$ T cells and were infected with WT-PbA or OVA-PbA. Seven days after the infection, these mice received splenocytes that were differentially labeled with CFSE and were left unpulsed ($CFSE^{low}$) or pulsed ($CFSE^{high}$) with OVAp. The spleen cells were analyzed 4 h later (Fig. 8). OVAp-coated target cells were specifically and almost completely cleared within 4 h in OT-I $CD8^+$ T cell-transferred OVA-PbA-infected mice. Peptide-pulsed targets were also significantly reduced in OT-I $CD8^+$ T cell-transferred WT-PbA-infected mice, although the levels of reduction were much less than those in OVA-PbA-infected mice. These effects were not seen in OT-I $CD8^+$ T cell-transferred uninfected mice or in the infected mice without OT-I $CD8^+$ T cell transfer.

Pathogenesis of $CD8^+$ T cells activated by malaria infection

To determine the role of $CD8^+$ T cells activated during malaria infection, B6, RAG2-KO, and RAG2-KO OT-I mice were infected with WT- or OVA-PbA (Fig. 9). B6 mice died 8–12 days after infection with WT- or OVA-PbA with clinical signs of cerebral malaria. Although the incidence of the cerebral malaria in B6 mice was relatively low (60–80%), it was within the range reported by Amani et al. (35). RAG2-KO mice did not develop cerebral malaria and survived >30 days after infection with WT- or OVA-PbA, consistent with previous studies indicating the requirement for $CD8^+$ T cells in the development of cerebral malaria (7–11).

RAG2-KO OT-I mice were resistant to WT-PbA infection, similar to RAG2-KO mice, and survived >30 days after infection, suggesting that nonspecific activation of $CD8^+$ T cells is not by itself harmful to the host. However, RAG2-KO OT-I mice showed levels of parasitemia higher than RAG-2 KO mice and three of five mice died 13–29 days after infection with OVA-PbA. Although statistical analysis of these data showed that the difference in survival time was not significant between RAG2-KO OT-I and RAG2-KO mice in this particular experiment, we think that it is likely due to the small number of the mice used in this experiment. We observed similar data in another set of experiments; six of seven RAG-2 KO OT-I mice died 15–29 days after infection with OVA-PbA, whereas none of RAG-2 KO mice died within 30 days after infection. Taken together, these data suggest that the activation of malaria-specific $CD8^+$ T cells, in the absence of a diverse adaptive immune system, could lead to the development of lethal pathogenesis during infection with blood stage PbA.

Discussion

This study indicated using a model malaria Ag, OVA, that malaria Ag can be presented to specific $CD8^+$ T cells by APCs in TAP-dependent cross-presentation during infection with PbA. The requirement of TAP for this pathway suggests that cross-presentation of malaria Ags involves the phagosome-to-cytosol pathway, in which Ags are exported to the cytosol after engulfment and are transported into the ER via TAP molecules, as reported for some other microorganisms such as *M. tuberculosis* and *T. gondii* (12, 13, 18, 19). Although infection with the malaria parasite might modulate the function of DCs or inhibit cross-presentation (30, 36–38), our study clearly indicates that APCs are able to cross-present malaria Ags that they have engulfed and activate specific $CD8^+$ T cells during the erythrocyte stage of malaria infection. Although we used a model Ag, OVA, it is likely that the endogenous malaria Ags are presented in a similar manner. The identification of natural CTL epitopes expressed in the erythrocyte stage of malaria parasites would aid our understanding of the role of $CD8^+$ T cells against the malaria blood stage.

In addition to Ag-specific response of $CD8^+$ T cells, we have found that Ag-nonspecific $CD8^+$ T cells could proliferate, show activation phenotype, express granzyme B, and gain CTL function when the host mice were infected with PbA, albeit at a lower level. A couple of possibilities might account for this nonspecific activation of OT-I $CD8^+$ T cells. First, OT-I $CD8^+$ T cells might directly recognize the PbA epitope by cross-reactivity of their TCR. We think that this possibility is unlikely, because OT-1 $CD8^+$ T cells were activated not only in B6, but also in TAP-KO hosts, which are defective in the phagosome-to-cytosol pathway of Ag presentation, suggesting that the activation of OT-I $CD8^+$ T cells in vivo by WT-PbA did not require TCR engagement. In addition, a similar activation-phenotype was observed in $CD8^+$ T cells of P14 TCR-transgenic mice as well as other RAG2-KO TCR-transgenic mice during infection with malaria parasites (Fig. 6 and unpublished observations). Second, host $CD8^+$ T cells might be activated by parasite products via interaction with their receptors other than TCR. Naive and activated $CD8^+$ T cells express a variety of pathogen-recognizing receptors including TLRs (39). Engagement of these receptors with ligands derived from parasites might modulate T cell function without TCR signaling. In particular, it is known that TLR2 is expressed on activated T cells and exhibits costimulatory function for TCR-stimulated T cells or can directly induce Th1 effector function (40, 41). Malaria parasites express GPI anchors that are recognized by TLR2 (42), thus possibly directly modulating the function of host T cells. However, the activation of naive $CD8^+$ T cells by TLR stimulation has not been reported. A third possibility is that $CD8^+$ T cells are activated by cytokine(s) produced by the innate immune system in response to PbA infection (43–45). Our study suggested that NK cells are involved in nonspecific activation of $CD8^+$ T cells. NK cells produce cytokines such as IFN-γ and TNF-α during malaria infection (31). Naive T cells can be activated by cytokines without TCR engagement, which has been termed the innate T cell activation pathway (46). Taken together, it is likely that cytokines produced by NK cells, in combination with products of malaria parasites, participate in Ag-nonspecific activation of $CD8^+$ T cells during infection with PbA.

We demonstrated that two types of $CD8^+$ T cells are activated during malaria infection: those specific for malaria Ag and activated by TAP-dependent Ag presentation; and those activated nonspecifically. In both types of activation, $CD8^+$ T cells express the activation phenotype and granzyme B and can develop into functional CTL, although the levels of the nonspecific activation are much lower than the specific activation. Our study suggested that $CD8^+$ T cells that are activated in an Ag-specific manner are involved in the pathogenesis of severe malaria. Highly activated OT-I $CD8^+$ T cells preferentially sequestered in the brain of B6 mice that were transferred with OT-I cells and infected with OVA-PbA (Fig. 3). In this experiment, however, it was unclear whether these cells were involved in the pathogenesis of cerebral malaria, since host B6 $CD8^+$ T cells were sufficient to cause cerebral malaria. In contrast, RAG2-KO OT-I mice that were infected with OVA-PbA showed early death when compared with RAG2-KO mice, suggesting that activation of OT-I $CD8^+$ T cells was pathogenic to the host, likely due to bystander mechanisms (Fig. 9). OVA-PbA-infected RAG2-KO OT-I mice showed more severe parasitemia and died later than B6 mice, suggesting that the death of RAG2-KO mice was not caused by cerebral malaria but may have been caused by other pathological processes associated with the infection. Taken together, these results suggest that the activation of malaria-specific $CD8^+$ T cells can be pathogenic to the host, but the development of cerebral malaria may require additional factors as has been discussed (10, 11). On the other hand, RAG2-KO OT-I mice showed a clinical course indistinguishable from RAG2-KO mice when infected with WT-PbA, suggesting that $CD8^+$ T cells that are activated in an Ag-nonspecific manner are generally not pathogenic to the host. Nonspecific activation of $CD8^+$ T cells, however, does not require TCR engagement and thus might include a pool of peripheral $CD8^+$ T cells that recognize various MHC class I-bound epitopes including self-Ag. Therefore, it remains possible that activation and CTL development of the self-reactive pool of peripheral $CD8^+$ T cells could lead to the destruction of tissue and might be involved in the pathogenesis of malaria. Further studies on the molecular mechanisms underlying the malaria-specific and nonspecific activation of $CD8^+$ T cells are important for expanding our understanding of protection against *Plasmodium* infection and of the pathogenesis of severe malaria.

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Disclosures

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Plasmodium pre-erythrocytic stages: what's new?

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The pre-erythrocytic (PE) phase of malaria infection, which extends from injection of sporozoites into the skin to the release of the first generation of merozoites, has traditionally been the 'black box' of the *Plasmodium* life cycle. However, since the advent of parasite transfection technology 13 years ago, our understanding of the PE phase in cellular and molecular terms has dramatically improved. Here, we review and comment on the major developments in the field in the past five years. Progress has been made in many diverse areas, including identifying and characterizing new proteins of interest, imaging parasites *in vivo*, understanding better the cell biology of hepatocyte infection and developing new vaccines against PE stages of the parasite.

The pre-erythrocytic phase

The pre-erythrocytic (PE) phase of *Plasmodium* infection starts with parasite injection by mosquitoes into the skin, ends with parasite invasion of the first red blood cells and mainly comprises parasite multiplication in the liver. Our understanding of the PE phase has long been limited by the lack of appropriate tools for studying the small numbers of parasites present in the mammalian host. This situation has changed recently with the development of powerful tools that have turned study of the PE phase into a dynamic and productive field, and we are starting to grasp a new complexity of this phase of infection. Gene identification in both sporozoites and liver stages, although still complicated by the few parasites produced by mosquitoes and the low parasite infectivity *in vitro*, has already identified many new products of interest. Systematic mutagenesis projects in several laboratories have elucidated important protein functions and uncovered new aspects of parasite behavior. The PE phase can now be tackled using powerful imaging tools that allow tracking of the tiny and highly motile sporozoites *in situ* and visualizing of liver stage development in real time. These technological developments have boosted our understanding of the basic biology of the parasite and of host–parasite interactions, but they have also impacted on the vaccine field with the construction of genetically attenuated parasites that confer protection against a sporozoite challenge in rodents.

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Gene identification in pre-erythrocytic stages

With the availability of the complete sequence of the genomes of human and rodent *Plasmodium* species, DNA microarrays and proteomic studies have been conducted on various stages of the parasite [1–3]. However, because the number of sporozoites that are necessary for microarray and mass spectrometry approaches are still difficult to gather, other techniques that demand less starting RNA material have also been used. Because sporozoites present in mosquito midguts are poorly infective to mammals, whereas those present in mosquito salivary glands are highly infective, genes upregulated in the salivary gland sporozoites have been particularly sought. One strategy relied on large-scale cDNA expressed sequence tag (EST) sequencing and on the comparison of the number of ESTs for individual genes generated by midgut or salivary gland sporozoites. A total of over 20 000 ESTs from *Plasmodium berghei* was sequenced (M. Yuda *et al.*, unpublished; EST data are available at EMBL [http://www.ebi.ac.uk/embl/], GenBank [http://www.ncbi.nlm.nih.gov/GenBank/index.html] and DDBJ [http://www.ddbj.nig.ac.jp/]), which uncovered several important genes such as *spect* (encoding sporozoite protein essential for cell traversal) [4], *spect2* [5] and *CelTOS* (encoding cell traversal protein for ookinetes and sporozoites) [6]. Suppressive subtractive hybridization was also used, and this technique identified 30 *Plasmodium yoelii* genes upregulated in salivary gland sporozoites compared with midgut sporozoites [7] and 25 *P. berghei* genes upregulated in salivary gland compared with blood-stage parasites [8]. Serial analysis of gene expression, which provides quantitative data on gene expression, was also performed on salivary gland sporozoites of *P. berghei* and identified 123 expressed genes, including 66 novel genes [9].

The liver stage is the parasite stage that is most refractory to gene-identification techniques, because this stage is present in only ~1% of the cells *in vitro*. The genes expressed in liver stages were identified mostly by cDNA sequencing, using *P. yoelii* parasites grown with or without cultured cells [10–12]. More recently, DNA microarray and proteomic analyses were performed on *P. yoelii* liver stages purified from mouse livers using fluorescent parasites and a cell-sorting technique [13].

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Identification of sequence-specific transcription factors

In silico analysis of promoter sequences is an important approach for elucidating the regulatory mechanisms of gene expression in organisms whose genome has been sequenced. However, only a few such studies have been undertaken in *Plasmodium*. In a recent *in silico* analysis of *cis*-elements of *Plasmodium* genes [14], a new algorithm for analysis of the AT-rich genome of *Plasmodium* was applied to a sporozoite-specific gene cluster of *P. falciparum,* and a six base pair sequence, CATGCA, was identified as a candidate regulatory element. This motif seems to be a binding sequence for the Apetala 2 (AP2) family of transcription factors [15]. Interestingly, a transcription factor belonging to the AP2 family, designated AP2-O, regulates gene expression in ookinetes by binding to a six base-pair sequence, TAGCTA, and activates invasion-related genes (M. Yuda *et al.*, unpublished). Moreover, another AP2-like transcription factor, designated AP2-SP, was identified as a sporozoite stage-specific transcription factor similar to AP2-O in ookinetes (M. Yuda *et al.*, unpublished). AP2-SP is localized in the nucleus of both midgut and salivary gland sporozoites and binds to the eight base-pair sequence TGCATGCA, which encompasses the sequence reported by Young *et al.* [14]. Disruption of the gene encoding AP2-SP in *P. berghei* inhibits the capacity of the parasite to form sporozoites inside oocysts. These studies thus suggest that transcription factors of the AP2 family have a central role in stage-specific gene expression in *Plasmodium*. If this is confirmed, promoter sequence analysis should become a powerful functional genomics approach for discovering groups of genes involved in a particular process.

The natural history of the sporozoite

Progress was also made in understanding how sporozoites parasitize their host in rodents. Once injected into the skin, sporozoites have a limited time of active motility for leaving the skin and moving on to the liver. Intravital imaging shows that *P. berghei* sporozoites are no longer motile in the mouse skin after 1–2 h [16,17], whereas PCR evidence indicates that *P. yoelii* sporozoites exit the skin in a slow trickle extending for up to 3 h after their inoculation [18]. Using their motility, some skin sporozoites invade blood capillaries [16,19] and terminate their journey in the liver. Other skin sporozoites invade lymphatic vessels and end up in the proximal draining lymph node [16,18], where most are rapidly found inside dendritic cells [16]. However, not all sporozoites leave the skin: a proportion of the sporozoites is still present in the skin once they are no longer capable of active motility; the proportion that remains in the skin probably varies with many factors, including the parasite species and the degree of vascularization at the site of inoculation. Other events during the sporozoite journey, such as the passage across endothelial barriers in the skin and in the liver, remain to be illuminated. For example, how sporozoites interact with Küpffer cells, the resident macrophages in the liver, is still controversial (reviewed in Ref. [20]). Another important challenge will be to image the behavior of *Plasmodium falciparum* or *Plasmodium vivax* sporozoites in human skin.

The finding, in rodents, that some sporozoites inoculated into the skin never reach the liver but remain at the injection site and its draining lymph node raises several important questions. First, it is known that an infective mosquito bite triggers the degranulation of mast cells [21] and the recruitment of neutrophils after only 20–30 min [22], but it is not known how sporozoites evade the host innate defense. Also, what is the impact of the skin and lymph node sporozoites on the host adaptive immune response? It was recently found that, after inoculation of irradiated sporozoites into the skin, naïve $CD8^+$ T cells are primed in the lymph node draining the site of inoculation by dendritic cells and that removal of the lymph node abrogates protection [23]. It is still unclear, however, where the parasite antigens are delivered to antigen-presenting cells. Are they directly presented in the lymph node via sporozoites that actively reach the node, or are they left in the skin as dead parasites or as material shed by sporozoites gliding in the skin matrix or through skin cells and secondarily presented to the lymph node? Answering these questions should help us to define better how protective immunity can be induced.

The enigma of host-cell traversal

Sporozoites have the capacity to breach host-cell plasma membranes and traverse (glide inside and through) cells [24], and, since 2001, the questions of when and why cell traversal occurs during sporozoite infection *in vivo* have been studied extensively. Initially, the view was that cell traversal was important in the liver by playing a dual activating role: first on the sporozoite final invasion of hepatocytes, based on a report that traversing host cells is required for the regulated exocytosis of invasion proteins, and second on liver-stage development, based on the finding that wounded hepatocytes release hepatocyte growth factor (HGF) that, in turn, is essential for parasite development and host-cell survival (reviewed in Ref. [25]). However, these conclusions were invalidated by the generation of several distinct parasite disruptants [4–6,26,27], which are impaired in host-cell traversal and yet all exhibit normal cell invasion and intracellular development, which clearly demonstrates that cell traversal is not required for sporozoite invasion of, or development in, hepatocytes.

More recent work on this topic still does not lead to a complete picture. First, it was found that incubation of sporozoites for 45 min in high K^+ buffer, which is thought to mimic the intracellular environment, led to an increase of the proportion of intracellular sporozoites [28], but whether such K^+ treatment reproduces the effect of host-cell traversal remains to be established. Second, intravital imaging showed that *P. berghei* cell-traversal-deficient sporozoites were arrested in the skin soon after inoculation [22], which provides direct evidence that the sporozoite traversal activity is important in the skin and facilitates encounters with dermal vessels. However, the cell types that are traversed by normal sporozoites in the skin remain unclear and could be both non-phagocytic (mainly dermal fibroblasts) and phagocytic cells [22]. Third, *in vitro* work indicated that sporozoites tend to migrate through cells that express undersulfated heparin sulfate proteogly-

cans (HSPG) but to invade cells covered with oversulfated HSPG, primarily hepatocytes, which suggests that sensing the sulfation level of heparan sulfates on host-cell surfaces is important for the sporozoite switch to cell invasion [29]. Although more work is needed for a definitive picture of the contribution of host-cell traversal in the sporozoite life, a new working model proposes that traversing cells is important for progressing from the skin to hepatocytes, but is dispensable for invading hepatocytes, and that cell invasion, and possibly apical regulated exocytosis, is activated upon cell contact. However, further experiments are needed to establish whether repression of the cell traversal activity, which is a prerequisite for parasite entry into a vacuole, and activation of cell invasion respond to the same signal(s), and whether signals other than heparan sulfates are involved in the switch from cell traversal to cell invasion.

Sporozoite invasion of hepatocytes

Similar to other invasive stages of Apicomplexa, sporozoites actively enter host cells by forming a junction with the host-cell surface and gliding through the junction inside a parasitophorous vacuole (PV). The molecular composition of this junction, however, remains poorly characterized, and just a few proteins involved in sporozoite entry have been identified. Although heparan sulfates are known to play a role, we still do not know whether a host-cell transmembrane receptor is involved. The contribution of the tetraspanin CD81, which was found to be important for *P. yoelii* and *P. falciparum*, but not *P. berghei*, sporozoite entry into hepatocytes [30], remains unclear, but CD81 does not seem to act as a receptor for the sporozoite [31]. On the parasite side, proteases are likely to play a crucial role in sporozoite invasion, as for *Plasmodium* merozoites and *Toxoplasma* tachyzoites, and the two major sporozoite surface proteins, circumsporozoite protein (CSP) and thrombospondin-related anonymous protein (TRAP), are indeed proteolytically processed before or during cell invasion [32,33]. In addition to CSP and TRAP, several new sporozoite surface proteins have been characterized by gene targeting. Among them are P36 and P36p/P52, members of the 6-Cys-domain protein family unique to *Plasmodium*, which are predicted to be secreted and glycosylphosphatidylinositol (GPI)-anchored proteins, respectively. Although the conclusions of several studies using either *P. berghei P36* or *P36p* single mutants [34,35] or a *P. yoelii P36* and *P36p* double mutant [36] differ, these studies indicate that P36 and P36p play a role in establishing a PV during sporozoite entry or maintaining the parasitophorous vacuole membrane (PVM) during parasite development. Another new player of unknown function during entry is the thrombospondin-related sporozoite protein (TRSP), a transmembrane protein that, similar to CSP and TRAP, contains a thrombospondin type I repeat (TSR) in its extracellular domain. Inactivation of the gene encoding TRSP in *P. berghei* causes only a partial decrease in sporozoite entry into hepatocytes [37].

Liver-stage development

The intravacuolar parasite then undergoes exo-erythrocytic schizogony to generate tens of thousands of merozoites. The first molecules essential for parasite survival inside the host cell have only recently been identified by mutagenesis. UIS3 [38] and UIS4 [39], encoded by genes previously identified by subtractive suppressive hybridization [7], are small PVM-associated transmembrane proteins that are necessary for early development of liver stages and parasite survival. UIS3 was found to interact directly with liver fatty-acid-binding protein (L-FABP), a cytoplasmic carrier of fatty acids in hepatocytes; this finding suggests a pathway for hijacking fatty acids from the hepatocyte [40]. Another new important parasite molecule is sporozoite asparagines-rich protein 1 (SAP1), a cytoplasmic protein, which is important for normal expression, apparently at a post-trancriptional level, of a variety of proteins, including UIS3, UIS4 and P52/P36p [41].

Live parasites prevent the death of their host cells by directly interfering with the apoptotic pathway of the host cell [42], and caspase-mediated DNA fragmentation, a hallmark of apoptotic cell death, is not observed in hepatocytes infected with *P. berghei* [43]. Late in development, however, when merozoites are formed, the parasite induces a non-apoptotic cell death of the host cell by activating cysteine proteases other than caspases [43,44]. The parasite proteins that trigger host-cell death might include the proteases of the serine repeat antigen (SERA) family [45]. The SERA proteases, which were shown to be involved in parasite liberation at other stages of the parasite life cycle [46,47], are produced mainly at the time of merozoite formation and appear to be fully processed at this stage and to translocate to the host-cell cytoplasm. Strikingly, the switch of the phosphatidyl serine (PS) moieties from the inner to the outer leaflet of the cell membrane, which is typically seen in dying cells and which triggers phagocytosis by macrophages, does not occur during liver-stage-induced host-cell death. The newly formed merozoites block the switch by taking up the Ca^{2+} released from internal stores in dying cells and thus inhibiting the Ca^{2+}-dependent enzyme that normally translocates PS. In addition, mature infected cells give rise to merozoite-filled vesicles, called merosomes, which bud off from infected cells and safely carry packs of merozoites into the liver sinusoids [43,48]. A recent study reports that merosomes can leave the liver and eventually release merozoites in the lungs [49].

It can be predicted that, to ensure its continuing development, the parasite secretes signaling molecules into the cytoplasm of the host cell. In erythrocytes infected with *P. falciparum*, targeting of parasite proteins across the PVM mainly depends on the signal motif *Plasmodium* export element (Pexel) [50,51]. A recent report suggests that CSP of the liver stages of *P. berghei* translocates into the host-cell cytoplasm using the Pexel motif and outcompetes nuclear import of the nuclear factor kappa B and other transcription factors by binding to importin α, which results in the inhibition of inflammatory responses [52]. It is probable that other parasite factors secreted into the host-cell cytoplasm that affect the physiology of the host cell will be found in the future.

Vaccine development against pre-erythrocytic stages

Although several methods, such as impregnated bednets, are being implemented to control malaria, and there are new formulations to treat the disease, the hope for malaria eradication still relies on the development of vaccines that are cheap, easy to administer and of very long duration (reviewed in Refs. [53,54]). The only vaccine in Phase III trials, RTS,S, which is based on a portion of CSP and developed by GlaxoSmithKline (reviewed in Ref. [55]), does not fulfill any of these criteria. Nevertheless, its potential is exciting. In several trials, including trials in children or adults in endemic areas, the efficacy was consistently between 30% and 50% [56,57], and its duration was up to 18 months [58]. In one case, vaccination reduced significantly the incidence of severe disease by nearly 60%. Protection correlates in general with the serum levels of antibodies to CSP that inhibit sporozoite infectivity, and perhaps with effector T cells against the liver stages. It is conceivable that the effectiveness of RTS,S could be improved either by boosting with a different CSP vector [59], or by incorporation of non-CSP protective antigens [60]. The efficacy of another PE vaccine, named ME-TRAP and consisting of a fusion between TRAP and a string of T-cell epitopes from six PE antigens, has been tested in Phase IIa and IIb trials in human volunteers in the UK and Africa. Several regimens of priming and boosting with DNA and pox vectors elicited significant T-cell responses, but the protective epitope(s) was not identified. In some vaccines, there was an increase in the pre-patent periods of infection after challenge, which implies that there were reductions in parasite liver loads and partial protection. Nevertheless, a Phase IIb vaccine trial failed to protect adults in the Gambia [61] or children in Kenya [62].

By contrast to the results discussed above that were obtained with subunit vaccines, immunization of rodents, monkeys and humans with sporozoites attenuated by irradiation leads to sterile immunity. Vaccination of humans with *P. falciparum* was achieved by the bite of irradiated infected mosquitoes. However, complete protection required ~1000 mosquito bites divided into five to ten doses over the course of one year [63]. Sterile immunity in rodents, achieved after intravenous injection of the parasites, also requires two or three booster injections, and protection decays in about six months. On the basis of these findings, it has been suggested that radiation-attenuated sporozoites (RAS) contained hundreds of protective T-cell antigens and that it would be very difficult, if not impossible, to mimic the protection mediated by RAS using subunit vaccines [64]. However, recent data [65] show that RAS vaccination of CSP transgenic mice that are unable to generate antibodies to or T cells specific for CSP (CSP transgenics X JhT mice) is significantly less effective (protection was reverted by more than 95% in BALB/c transgenic mice and by ~50% in C57BL/6 transgenic mice), suggesting that RAS contain just a few T-cell-protective antigens. Nevertheless, boosting the CSP transgenic mice twice with RAS led to sterile immunity. The nature of these powerful non-CSP protective T-cell antigens is unknown, and their identification is an important priority for PE vaccine development.

A much-highlighted recent achievement was the generation of genetically attenuated sporozoites (GAS). In a similar way to RAS, the growth of GAS in which *UIS3*, *UIS4* and/or *P36*/*P36p* are inactivated is arrested during their liver-stage development, and this leads to sterile immunity in mice (reviewed in Refs [66,67]), although the development of UIS3- and UIS4-deficient parasites appears to stop earlier than that of RAS. Whether GAS- and RAS-induced protective immunities are similar is unclear. As in RAS, the efficacy of GAS-induced protection in rodents has been studied mostly after intravenous immunization and one or two boosters are required to achieve sterile immunity. In one publication, a single intravenous injection of *P. yoelii* UIS3-deficient parasites led to complete protection [68], but others were unable to reproduce these results (K. Kumar, unpublished). Whether protection depends on parasite persistence in the host is also controversial. Whereas earlier studies had indicated that RAS-induced protection required parasite persistence [69], this is not supported by more recent work on RAS, which found that the maximum activation of antigen-specific $CD8^+$ T cells occurs within the first 8 h after immunization and lasts for ~48 h [70]. For GAS, protection induced by *P. berghei* UIS3-deficient parasites was found to depend on parasite persistence [71], but protection induced by *P. yoelii* UIS3- or UIS4-deficient parasites was not [68]. Finally, although work on GAS indicates that they induce similar mechanisms of protection to those induced by RAS, and they are mediated by antibodies and $CD4/CD8^+$, IFN-γ-producing T cells, recent work on RAS questions the role of $CD8^+$, IFN-γ-producing T cells in protection [72]. Clearly, we still do not understand how live parasites that are attenuated by radiation or gene targeting protect their host.

How realistic is the possibility of vaccinating humans in endemic areas with *P. falciparum* RAS or GAS? Although other authors have expressed optimistic views and enthusiasm for this approach [66,67,73], the obstacles are formidable. They include the need to store the parasites frozen until used, to guarantee the absence of viral and bacterial contaminants and to ensure the reproducibility of different lots of parasites. Even more crucially, and assuming that the frozen–thawed sporozoites have properties similar to those delivered by infected mosquito bites, the intradermal or intramuscular delivery is likely to remain less effective than intravenous injection. In addition, many booster injections will be necessary to achieve sterile immunity, which makes it logistically impractical for endemic areas, and the duration of immunity will be measured in months, not years. Nonetheless, whatever the future of GAS as vaccines for humans, GAS in rodents will constitute powerful tools to dissect the basis of protection against the PE stages of the parasite.

Conclusion

It is clear from this brief overview that the PE field has been active in the past five years and that paradigms are being challenged or are changing. We now view the PE phase as integrating many host–parasite interactions in different tissues in addition to the central parasite multiplication inside hepatocytes, and the newly available com-

bination of *in silico*, molecular genetic and imaging tools multiplies the questions that can be addressed. Whatever the aim, deconstructing the biology of the parasite, the interactions with the host or the host immune response, or identifying new protective antigens and vaccine vectors, we expect the PE field to continue to generate much excitement.

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NOTE *Parasitology*

A PCR-Based Epidemiological Survey of *Hepatozoon canis* in Dogs in Nigeria

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ABSTRACT. The prevalence of *Hepatozoon canis* infections in dogs in Nigeria was surveyed using molecular methods. DNA was extracted from blood samples obtained from 400 dogs. A primer set that amplified the *Babesia canis* 18S rRNA gene, which has high similarity to the *H. canis* 18S rRNA gene, was used for the PCR. As a result, samples from 81 dogs (20.3%) produced 757 bp bands, which differed from the 698 bp band that corresponded to *B. canis* infection. The sequence of the PCR products of 10 samples were determined, all of which corresponded with the *H. canis* sequence.

KEY WORDS: *Hepatozoon canis*, Nigeria, PCR.

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Hepatozoon canis is a protozoan that is transmitted by the ixodid *Rhipicephalus sanguineus*. *H. canis* is present in southern Europe, Asia, Africa and Latin America [2–4, 8, 9]. This parasite is transmitted to dogs by the ingestion of ticks containing mature oocysts. In infected dogs, gametocytes are present within peripheral blood leukocytes. *H. canis* infections range from an asymptomatic type to a lethal type in which symptoms such as fever, anemia, emaciation and debility are displayed. Concurrent infections with pathogens such as *Babesia canis* and *Ehrlichia* spp. may aggravate the clinical condition of dogs infected with *H. canis* [8].

Diagnosis of canine hepatozoonosis is usually accomplished by microscopic examination of stained peripheral blood smears for the presence of gametocytes within neutrophils. However, it is sometimes difficult to detect the parasite when the number of circulating gametocytes is low. Serological tests such as an indirect fluorescent antibody test and an enzyme-linked immunosorbent assay have been developed for the diagnosis of canine hepatozoonosis [1, 7, 8, 12]. Recently, molecular methods have been used to detect *Hepatozoon* spp. Molecular PCR methods are more sensitive and specific for pathogens in peripheral blood and arthropod vectors than other methods [2, 8]. Moreover, PCR analysis followed by sequence analysis can be used for phylogenic characterization of *Hepatozoon* isolates [2]. These molecular methods can play an important role in the diagnosis of canine hepatozoonosis and research into its distribution and prevalence.

Molecular methods have been used worldwide to conduct epidemiological studies of canine hepatozoonosis [1, 2, 8, 10]. A study demonstrated that *H. canis* and its arthropod vector, *R. sanguineus*, are present in the Sudan [10]. As *R. sanguineus* is thought to be widely distributed throughout Africa, it is possible that *H. canis* is widespread on this continent. *H. canis* was reported to be present in Nigeria, in the Midwest of Africa [5], but this has not been validated by an epidemiological survey in which molecular methods have been used.

In a previous study, we surveyed the prevalence of *B. canis* infection among 400 dogs in Nigeria using nested PCR and primer sets that amplified the *B. canis* 18S rRNA gene [11]. *B. canis* infection resulted in a 698 bp band after nested PCR. Some of these samples also produced a 757 bp band, which was larger than the *B. canis* band (698 bp). Therefore, the 757 bp PCR products were used for nucleotide sequence determination. The sequences of samples were shown to correspond to that of *H. canis* 18S rRNA genes (99.3%, GenBank accession no. AF176835). In the present study, we analyzed the sequences of these PCR products and investigated the incidence of *H. canis* infection in dogs in Nigeria.

Peripheral blood samples from 400 dogs were obtained randomly from many parts of Nigeria between October 2004 and August 2005. Each 200 μL of blood sample was fixed on an FTA Card (Whatman, UK) and stored at room temperature in the dark. DNA was extracted from blood on the FTA Cards using the Whatman FTA Card DNA Isolation kit (Whatman).

Primers for the PCR were based on a fragment of the gene that encodes *B. canis* 18S rRNA (GenBank accession no. AY072925, AY072926, L19079) [11]. Forward primer B18S-1 (5'-GGGAGGTAGTGACAAGAAA- 3') and reverse primer B18S-2 (5'-TTCCCCGTGTTGAGTCAAA-3') were used for the PCR [6, 11]. The sequences of the *B. canis* and *H. canis* 18S rRNA genes were similar to those of the respective primer sets (Table 1).

The DNA extracted from the blood samples was added to

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Table 1. Sequences of the B18S-1 and B18S-2 primers and *H. canis* 18S rRNA gene

Primer	Primer sequence (5'–3')	*H. canis* nucleotide sequence
B18S-1	GGGAGGTAGTGACAAGAAA	GAGAGGTAGTAACAAGAAA
B18S-2	TTCCCCGTGTTGAGTCAAA	TTTCCCGTGTTGAGTCAAA

Table 2. Sex and age of dogs positive for *H. canis*

		No. of examined	*H. canis* positive
	Total	400	81 (20.3%)
Sex	Male	169	34 (20.1%)
	Female	227	47 (20.7%)
	ND*	4	0
Age (yr)	0–2	292	58 (19.9%)
	3–5	77	19 (24.7%)
	6–8	17	2 (11.8%)
	9–	10	2 (20.0%)
	ND*	4	0

*ND: no data.

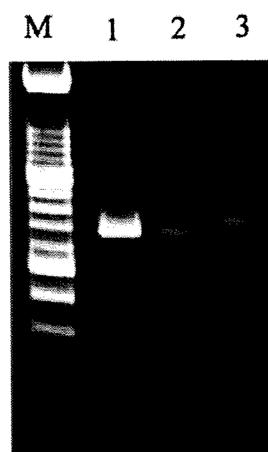

Fig. 1. Results of nested PCR analysis using primer sets that amplified *B. canis* 18S rRNA. Lane M, molecular size marker; lane 1, *B. canis rossi*; lane 2, *B. canis vogeli*; lane 3, *H. canis*.

a reaction mixture containing 10 pmol each of primers B18S-1 and B18S-2, 5 U/μL of AmpliTaq Gold DNA polymerase (Applied Biosystems, US), 2.0 mM dNTP (Applied Biosystems), 10 × PCR Gold Buffer (150 mM Tris-HCl [pH 8.0], 500 mM KCl) (Applied Biosystems) and 25 mM $MgCl_2$ Solution (Applied Biosystems), and adjusted to a volume of 25 μL with Ultra Pure Distilled Water. Amplification conditions were as follows: 95°C for 10 min, 30 cycles of denaturation at 94°C for 30 sec, primer annealing at 55°C for 2 min and amplification at 72°C for 2 min, followed by final extension at 72°C for 5 min. PCR products were separated by electrophoresis on 1.5% TBE agarose gel and stained with ethidium bromide.

The amplified DNA was cloned into a pCR® 2.1 - TOPO® vector using a TOPO TA Cloning® Kit (Invitrogen, US) and the sequences were determined using the B18S-1 or B18S-2 primers and an ABI PRISM® 310 genetic Analyzer (Applied Biosystems). The sequences were analyzed using Genetyx® Version. 8 (Genetyx, Japan).

The PCR analysis showed that 81 of 400 samples (20.3%) contained fragments of the 757 bp PCR product which differed from the 698 bp band that corresponded to *B. canis* infection (Fig. 1). The PCR products of 10 samples selected at random from the 81 samples that produced fragments of the 757 bp band were used for nucleotide sequence determination (GenBank accession no. AB365071). The sequences of all 10 samples were shown to correspond to that of *H. canis*, indicating that all 81 samples were positive for *H. canis*. The sexes and ages of dogs infected with *H. canis* are shown in Table 1. Of the 81 dogs infected with *H. canis*, 34 were male and 47 were female. The ages of the infected dogs range from 5 weeks to 11 years. There was no correlation between the incidence of *H. canis* and the age and sex of the dogs. In none of the samples, gametocytes were detected in neutrophils by microscopic examination of Giemsa-stained peripheral blood smears.

In Nigeria, Ibrahim *et al.* [5] studied 18 dogs that exhibited clinical signs of *H. canis* infection and detected *H. canis* gametocytes in myeloperoxidase-deficient neutrophils obtained from these dogs. However, an extensive investigation of the prevalence of *H. canis* infection in Nigeria has not been performed using molecular methods. In the whole of the African continent, the only epidemiological study to have been performed using PCR was undertaken in the Sudan [10]. In the Sudan study, 33 of 78 dogs (42.3%) were infected with *H. canis*, and among these, three dogs were also infected with *B. canis* [10]. Our study showed that in Nigeria, 81 of 400 dogs (20.3%) were infected with *H. canis*, a much lower incidence of infection than in the Sudan. The incidence of *B. canis* infection in dogs is also lower in Nigeria than in the Sudan [10, 11]. *R. sanguineus* is the dominant tick species in the Sudan [10], and transmits both *H. canis* and *B. canis vogeli*. The disparity between the results of our study and those of the Sudan study may have been caused by differences in sizes of tick populations or their distribution.

The 400 blood samples used for this nested PCR study were identical to those used in our previous study of *B. canis* infection [11]. Of the 400 dogs, 81 samples produced a 757 bp band, and nine samples produced a 698 bp band. The sequences of these PCR products corresponded to those of *H. canis* and *B. canis*, respectively. Of the nine samples that

produced a 698 bp band, eight were identified as *B. canis* rossi, and one as *B. canis* vogeli [11]. Two samples were positive for both *H. canis* and *B. canis*. The primers used in this study made simultaneous diagnosis of both *H. canis* and *B. canis* infection possible. *H. canis* causes relatively mild symptoms in dogs, but the clinical condition of dogs infected with *H. canis* may deteriorate when they are concurrently infected with another pathogen [8]. In Nigeria, the presence of *R. sanguineus* may result in concurrent infections with *H. canis* and *B. canis*, because it is a vector for both these pathogens.

In conclusion, our molecular study revealed the infection of *H. canis* with dogs in Nigeria and demonstrated the existence of concurrent infection with *B. canis*. As infected dogs that do not display symptoms may be present throughout the African continent, it is important to detect carrier dogs and to control vector tick infestations.

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NOTE *Parasitology*

Molecular Survey of *Babesia canis* in Dogs in Nigeria

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ABSTRACT. An epidemiological study of *Babesia canis* in dogs in Nigeria was performed. Four hundred blood samples collected from dogs in Nigeria were investigated using nested PCR and sequence analysis. On nested PCR screening, nine samples (2.3%) produced a band corresponding to a 698-bp fragment indicative of *B. canis* infection. Sequence analysis of the PCR products identified eight samples (2.0%) as *B. canis rossi* and the ninth (0.3%) as *B. canis vogeli*. This is the first report of the prevalence of *B. canis rossi* and *B. canis vogeli* in dogs in Nigeria.

KEY WORDS: *Babesia canis*, Nigeria, PCR.

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Babesia canis is a tick-transmitted hemoprotozoan parasite that induces anemia, fever, jaundice, hemoglobinuria, and sometimes fatal symptoms in dogs. *B. canis* is classified into three subspecies: *B. canis rossi*, *B. canis vogeli*, and *B. canis canis* [5, 10, 11]. The arthropod vectors of *B. canis rossi*, *B. canis vogeli*, and *B. canis canis* are *Haemaphysalis leachi*, *Rhipicephalus sanguineus*, and *Dermacentor reticulatus*, respectively [5, 10, 11].

A diagnosis of canine babesiosis is made by the detection of parasites in a peripheral blood smear under a microscope or by serological tests such as an indirect fluorescent antibody test or enzyme-linked immunosorbent assay. Compared with these methods, molecular analysis using PCR has greater sensitivity and specificity in the detection of pathogens [1, 4, 8, 9, 11]. Thus, the PCR method makes it possible to diagnose babesiosis in the blood samples of dogs that show very low parasitemia, as in the early stages of infection, or in dogs with no symptoms. Moreover, it is possible to differentiate species or subspecies of pathogens by sequence analysis following PCR [2, 9–11].

Throughout the African continent, epidemiological studies of canine babesiosis using molecular methods have been reported only in South Africa and the Sudan, and *B. canis rossi* and *B. canis vogeli* have been shown to be distributed in both countries [10, 11]. *B. canis vogeli* is thought to be distributed in large parts of the tropical and subtropical regions of all continents in which its vector ticks exist [12]. Therefore, it is anticipated that this parasite is distributed widely throughout the African continent. However, the prevalence of *B. canis* infections in dogs in other parts of Africa is unknown. To determine the incidence and identify subspecies of *B. canis* in dogs in Nigeria, located in the midwest of Africa, we performed an epidemiological study using PCR and sequence analysis.

Peripheral blood samples from 400 dogs were obtained randomly from many parts of Nigeria between October 2004 and August 2005 (Table 1). Each 200 μl of blood was fixed on an FTA Card (Whatman, UK) and stored at room temperature in the dark. DNA was extracted from the blood samples on the FTA Cards using the Whatman FTA Card DNA Isolation Kit (Whatman).

Primers for the first PCR and nested PCR were designed based on a fragment of the gene that encodes *B. canis* 18S rDNA (GenBank accession nos. AY072925, AY072926, and L19079). The forward primer B18S-F (5'-TGGTTGATCCTGCCAGTA-3') and the reverse primer B18S-R (5'-CTTCTCCTTCCTTTAAGTGA-3') were used in the first amplification reaction [7]. Primers B18S-1 (5'-GGGAGGTAGTGACAAGAAA-3') and B18S-2 (5'-TTCCCCGTGTTGAGTCAAA-3') were used in the nested PCR.

The DNA extracted from the blood samples on the FTA Cards was added to a reaction mixture containing 10 pmol of each primer B18S-F and B18S-R, 5 U/μl AmpliTaq Gold

Table 1. Sex and age of dogs examined

		No. of examined
	Total	400
Sex	Male	169
	Female	227
	ND*	4
Age (yr)	0–3	336
	4–7	46
	≥ 8	14
	ND*	4

* ND: not data.

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DNA polymerase (Applied Biosystems, U.S.A.), 2.0 mM dNTPs (Applied Biosystems), 10 × PCR Gold Buffer (150 mM Tris-HCl [pH 8.0], 500 mM KCl; Applied Biosystems), and 25 mM $MgCl_2$ (Applied Biosystems). The reaction mixture was adjusted to 25 μl with ultrapure distilled water. The amplification conditions were as follows: 95°C for 10 min; 30 cycles of denaturation at 94°C for 30 s, primer annealing at 55°C for 2 min, and amplification at 72°C for 2 min; followed by a final extension at 72°C for 5 min. The nested PCR was performed using primers B18S-1 and B18S-2 under the same conditions as the first PCR. The PCR products were separated by electrophoresis on 1.5% TBE agarose gel and stained with ethidium bromide. The expected size of PCR product for *B. canis* 18S rDNA is a 698 bp fragment after nested PCR.

The amplified DNA was purified with the QIAquick® PCR Purification Kit (Qiagen, Germany), and the sequences were determined directly using the B18S-1 or B18S-2 primer with the ABI PRISM® 310 Genetic Analyzer (Applied Biosystems). The sequences obtained were analyzed with Genetyx® version 8 (Genetyx, Japan).

In the first PCR reaction using the primers B18S-F and B18S-R, no positive bands have been detected in all samples. In the nested PCR using the primer set designed in this study, nine samples (2.3%) in 400 samples showed a band of 698 bp fragment which is expected size for *B. canis* 18S rDNA. The sequences of these nine PCR products were determined (*B. canis rossi*, AB303071-AB303075; *B. canis vogeli*, AB303076). Eight samples (2.0%) showed high sequence similarity to the 18S rRNA gene of the *B. canis rossi* isolate from dog 44 (DQ111760) from the Sudan at both the nucleotide (99.1%–99.8%) and predicted amino acid levels (98.7%–99.1%). Another sample (0.3%) was similar in its sequence to a *B. canis vogeli* 18S rRNA gene from Italy (AY072925) at both the nucleotide (99.6%) and predicted amino acid levels (98.7%). Moreover, this sequence also showed partial sequence homology to a *B. canis vogeli* 18S rRNA gene isolated in South Africa (AF547387) at both the nucleotide (99.6%) and predicted amino acid levels (98.7%), and to the sequence of a *B. canis vogeli* 18S rRNA gene from the Sudan (DQ111765) at both the nucleotide (99.6%) and predicted amino acid levels (98.9%).

In this study, eight of 400 dogs (2.0%) were positive for *B. canis rossi* and one dog (0.3%) was positive for *B. canis vogeli*. In contrast, the incidence of *B. canis* in dogs in South Africa and the Sudan were 14.8% and 9.0%, respectively [10, 11]. Thus, the infection rates of *B. canis rossi* and *B. canis vogeli* are lower in Nigeria than those in South Africa or the Sudan. *B. canis rossi* and *B. canis vogeli* are known to be transmitted by ticks, and the rates of tick infestations in dogs in South Africa and the Sudan are reported to be 72.2% and 78.2%, respectively [6, 11]. The arthropod vectors of *Babesia* spp., such as *H. leachi* and *R. sanguineus*, have been confirmed to occur widely in South Africa and the Sudan [6, 10, 11]. A lower rate of tick infestation in dogs in Nigeria might contribute to their lower rates of infection with *B. canis rossi* and *B. canis vogeli*. However, there has been no report of the occurrence of the tick vectors of *Babesia* spp., such as *H. leachi, R. sanguineus*, and *D. reticulatus*, in Nigeria. The rate of dogs infested with ticks should be investigated, and the transmission of *B. canis rossi* and *B. canis vogeli* by these ticks in Nigeria confirmed.

Table 2. Dogs positive for *Babesia canis* in Nigeria

Dog no.	Breed	Sex	Age (yr)	Subspecies
2	Alsatian	Male	0.2	rossi
8	Rottweiler	Female	0.3	rossi
23	Local	Female	1	rossi
25	Doberman	Male	3	rossi
127	Local	Male	2	rossi
135	Local	Female	1	rossi
266	Alsatian	Male	2	vogeli
354	Local	Female	3	rossi
398	Alsatian	Male	0.4	rossi

In this study, young dogs under three years were positive for *B. canis* (Table 2). Moreover, it is noteworthy that three samples from puppies under six months were also positive (Table 2; dogs 2, 8, and 398). Fukumoto *et al.* [3] have demonstrated transplacental infection of *B. gibsoni* in dogs. The *B. canis* infections of young dogs recorded in this study suggest that this parasite is also vertically transmitted from dogs to their puppies. However, there are few samples from old dogs, and more samples from dogs over four years should be examined.

In conclusion, the primers designed in this study are useful for the detection of *B. canis*. PCR with these primers and sequence analysis have demonstrated that *B. canis rossi* and *B. canis vogeli* exist in dogs in Nigeria.

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Ⅲ．平成21年度　総括・分担研究報告

厚生労働科学研究費補助金（新型インフルエンザ等新興・再興感染症研究事業）
総括研究報告書

遺伝子増幅 RPA 法に基づいた媒介蚊における迅速簡便病原体検出法の開発

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研究要旨　　マラリア、西ナイル熱、デング熱等の蚊媒介性の再興感染症は世界的に大きな脅威となっている。本研究では、これらの感染症の本邦への侵入防除に寄与するため、等温遺伝子増幅法による迅速・簡便病原体検出法の確立を目的として以下の項目に分け遂行した。(1) 原虫感染モデルとしてハマダラカ-齧歯類マラリア原虫感染モデル、ウイルス感染モデルとしてヤブカ-フロックハウスウイルス感染モデル、蠕虫感染モデルとしてヤブカ-犬糸状虫感染モデルを用いた、蛍光プライマーによるマルチプレックス等温遺伝子増幅法の開発および、病原体検出評価に成功した。(2) プライマーに蛍光標識を施し、これらの標識物に対する抗体を用いたイムノクロマトストリップを作製し、簡便に遺伝子増幅産物を検出可能な系を構築することに成功した。(3) 病原体媒介蚊における殺虫剤耐性の検出法の開発のため、等温遺伝子増幅法による SNP 検出の有用性を検討した。(2) 熱帯熱マラリア流行地域である西アフリカ・ブルキナファソ国における蚊生息フィールドでの蚊サンプル採集を実施し、病原体保有率および殺虫剤耐性蚊の生息状況調査に成功した。以上の研究により、病原体媒介蚊における病原体検出に対する総合的な厚生労働行政施策を策定するための科学的基盤を進展させた。

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A．研究目的

マラリア、西ナイル熱、デング熱等の蚊媒介性の再興感染症は世界的に大きな脅威となっている。本研究では、これらの感染症の本邦への侵入防除に寄与するため、等温遺伝子増幅法による迅速・簡便病原体検出法の確立を目的とした。本研究で対象とする感染症では、たった一つの病原体の侵入でも人への感染成立が可能なため、高感度かつ特異的な検出法が必要であり、これらの特性に優れた PCR 法が現在の主流となっている。しかしながら PCR 法は高度な設備を必要とし、汎用性に問題がある。近年、PCR 法などの諸問題を解決する遺伝子増幅手法として、LAMP 法などに代表される等温遺伝子増幅法が開発された。等温遺伝子増幅法は PCR 法と同様にプライマーによって反応がおこなわれる。遺伝子の増幅は一定温度下で 60 分程度と極めて短時間であり、特異性は PCR に準じ、また増幅産物をイムノクロマト法により目視による判定が原理的に可能であり、極めて迅速かつ簡便性に優れた手法と考えられる。また、一回の反応で各種病原体遺伝子を検出するマルチプレックス化により、数種類の病原体を短時間で一度に検出することが可能である。

遺伝子増幅による新規の病原体検出系の開発にあたっては、プライマーの設計など反応条件の至適化が必要なため、開発着手から実用化までに長大な時間を要する。LAMP 法など等温遺伝子増幅法の優れた点として、ひとつの標的遺伝子に対して 4 つないしは 6 つのプライマーの組み合わせを用いるため、比較的

短時間に特異性の高い条件の設定名可能で、各種病原体への応用を迅速におこなうことができる。さらに、LAMP 法はラダー状の増幅遺伝子産物が大量に生じるため、プライマー標識等による簡便検出などの更なる解析に応用可能である。

上記のように、等温遺伝子増幅法は既存の方法の持つ問題点を克服し、様々な優れた特性を有する手法である。LAMP 法等の等温遺伝子増幅原理の病原体検出法としての応用開発研究は、我が国を含めた世界各国に於ける新規病原体サーベイシステムとして、多大なる貢献をもたらすものと考えられる。

B．研究方法

本研究は、世界的な驚異となっている蚊媒介性再興感染症、特にマラリア、西ナイル熱、デング熱などの日本への侵入を防除するため、等温遺伝子増幅法を応用した迅速かつ簡便な病原体検出システムの開発を目指すものである。

研究計画の概略は以下の通りである。齧歯類マラリア原虫とフロックハウスウイルスを用いた媒介蚊-病原体感染モデルを用いることで等温遺伝子増幅法による蚊からの病原体検出システムの有効性を検証、反応条件等の至適化をおこなう。次に、本来の対象病原体である熱帯熱マラリア原虫、西ナイル熱ウイルス、デング熱ウイルスなどを検出可能な等温遺伝子増幅法の開発をおこなう。また、イムノクロマト法による増幅産物検出法を確立し、簡便な検定結果の判定法を開発する。さらに、多種の病原体を同時に検出可能な「マルチプレックス等温遺伝子増幅法-イムノクロマト法」の開発をおこなう。その後、日本国内および諸外国より収集した蚊サンプルを用いて、等温遺伝子増幅法の実用性の評価、さらに世界的な蚊媒介性再興感染症の正確な汚染状況を把握することを最終目標とする。本研究課題は三ヶ年の計画から成り、平成 21 年度の計画は以下に述べる。

平成 19 年度より開始した本研究課題では、これまでに原虫感染モデルとして齧歯類特異的なマラリア原虫を用いた「ハマダラカ-マラリア原虫感染モデル」、ウイルス感染モデルとして「ヤブカ-フロックハウスウイルス感染モデル」の樹立を達成し、本年度も研究を順調に遂行した。上記研究基盤をもとに、平成 21 年度では、媒介蚊-病原体感染モデルを用いた等温遺伝子増幅法法の評価、マルチプレックス等温遺伝子増幅法の開発と、等温遺伝子増幅法増幅産物による SNP 検出法の確立を目標とした。

以上の等温遺伝子増幅法を用いた病原体迅速・簡便検出法として応用開発研究により、媒介蚊の正確な病原体保有状況をサーベイすることが可能となり、我が国の病原体管理体制強化に大きく寄与するものと考えられる。

これらの研究は、以下の分担研究課題として遂行され、本研究の根幹として各課題が連携しながら進めた。

(1) 等温遺伝子増幅法による病原体媒介蚊の殺虫剤耐性変異の SNP 検出（研究分担者：嘉糠洋陸）

(2) 蛍光マルチプレックス等温遺伝子増幅法の開発（研究分担者：福本晋也）

(3) 等温遺伝子増幅法テンプレート用病原体の作製（研究分担者：下島昌幸）

(4) 等温遺伝子増幅産物の検出に向けたイムノクロマト・ストリップ作成法の確立（研究分担者：平田晴之）

(5) ネズミマラリア原虫を用いたヒトマラリア原虫感染モデルの構築（研究分担者：油田正夫）

本年は 3 年計画研究の最終年度であり、進捗状況および研究成果を会議等で確認・情報交換し、当該研究成果に立脚して随時研究課題を検討・修正した。

（倫理面への配慮）

動物実験に関しては、「実験動物の飼養及び保管等に関する基準（昭和 55 年総理府告示第 6 号）」に則り、動物実験を行う施設ごとの「動物実験に関する基本方針」や「動物実験施設管理運営規定」等を十分に遵守して研究を遂行した。

遺伝子組換え実験および微生物利用実験について、前者は文部科学省「遺伝子組換え生物等の使用等の規制による生物の多様性の確保に関する法律」及びこれにもとづく政省令・告示に示される基準に適合し、かつ所属機関の承認を得て遂行する。後者についても、各研究機関の「病原体等安全管理規程」等にもとづき、実施した。

疫学研究に関わる試料採集等の研究遂行にあたっては、我が国の文部科学省・厚生労働省が共同で作成した「疫学研究に関する倫理指針（平成 14 年 6 月 17 日）（平成 16 年 12 月 28 日全部改正）（平成 17 年 6 月 29 日一部改正）」に従い、流行地における疫学調査研究にも同様にあてはめた。それぞれの国・地域に

おける対象となる住民の不利益になることの無いように最大限の配慮を注いだ。

C．研究結果

(1) 等温遺伝子増幅法による病原体媒介蚊の殺虫剤耐性変異の SNP 検出

1． AS-LAMP 法によるハマダラカ殺虫剤耐性 SNP の検出

殺虫剤耐性をもたらす *kdr* 遺伝子の変異にはいくつかの種類が知られている。そのうち、西アフリカ型の変異では、*kdr* 遺伝子の 1104 番目の塩基がＡからＴに置換されることにより、該当するアミノ酸がロイシンからフェニルアラニンに変化する。そこで、野生型と西アフリカ型のこの一塩基の差異を検出するように FIP 及び BIP プライマーを設計し、AS-LAMP 反応に供与した。その結果、テンプレートとして（1）*kdr* 遺伝子の cDNA を持つプラスミド、および（2）ハマダラカ DNA を用いた双方において、特異的な増幅に成功した。特にハマダラカにおいては、西アフリカ型と野生型のヘテロの遺伝子型を有するサンプルも区別可能なことが判明し、AS-LAMP 法の高い特異性が示された。また、従来の SNP 検出のための AS-PCR 法との比較を実施したところ、ほぼ同等の性能か、特異性についでは PCR を上回る結果を得た。また、この AS-LAMP 法を用いて、ブルキナファソから採集した蚊サンプル群について *kdr* 遺伝子の性状解析を実施したところ、３地点において 10-26％の蚊が殺虫剤耐性となっていることが明らかとなった。

(2) 蛍光マルチプレックス等温遺伝子増幅法の開発

1． 蛍光標識プライマーを用いた等温遺伝子増幅反応の検討

蛍光標識プライマーが LAMP 法による等温遺伝子増幅反応に影響を与えるのか否かを検討した。1、10、または 100 個のマラリア感染赤血球より抽出した DNA、もしくは 1、10、または 100 ミクロフィラリア虫体より抽出した DNA をテンプレートとして蛍光標識プライマーを用いた LAMP 法の増幅効率を検証した。マラリア感染赤血球の場合、蛍光非標識プライマーを使用した場合、検出限界は $1x10^2$ の感染赤血球由来 DNA であった。一方、標識プライマーを使用した場合、検出感度は $1x10^2$ 感染赤血球由来 DNA であり、検出感度の低下は認められなかった。フィラリアの場合も同様に非標識プライマーを用いた場合、標識プライマーを用いた場合、の比較を行ったところ検出限界に差は無く、一個体のミクロフィラリア DNA 由来サンプルを検出可能であり、プライマー標識による検出限界の低下は認められなかった。

単一チューブ内の反応においてマラリア原虫、フィラリア双方の DNA を検出するマルチプレックス化を検証するため、フィラリア検出プライマー、マラリア検出プライマーを混合し、感染赤血球由来 DNA、またはミクロフィラリア由来 DNA を検体として LAMP 法に供した。その結果、混合標識プライマーの使用においても特異的に病原体 DNA を検出することが可能であった。しかしながら検出限界の低下が認められ、50 マラリア感染赤血球由来 DNA が必要であった。

蛍光標識プライマーによる等温遺伝子増幅法が病原体感染蚊由来 DNA サンプルに対しても同様に適用可能なのかを確認するため、オーシストステージのマラリア原虫感染ハマダラカ、またはフィラリア第 2 期幼虫感染ネッタイシマカ由来サンプルをテンプレートとして LAMP 法に供した。感染蚊を実体顕微鏡下で解剖し感染病原体数を確認した後に、LAMP 反応に供した。マラリア原虫オーシスト数は１ハマダラカあたり 1～974 個の範囲内でありであり、１ネッタイシマカあたりのフィラリア第 2 期幼虫数は 1～7 虫体であった。これらのサンプル由来 DNA を両病原体検出プライマーがミックスされた反応系でそれぞれ LAMP 法に供したところ、マラリア原虫においては 19 オーシストが感染しているハマダラカ、フィラリアにおいては、1 虫体の第 2 期幼虫が感染しているネッタイシマカから病原体 DNA を検出可能であることが明らかになった。

(3) 等温遺伝子増幅法テンプレート用病原体の作製

1．C 型レクチンによる感染性西ナイルウイルス様粒子の感染増強作用の機序

感染性西ナイルウイルス様粒子の感染は、昨年度報告したように、C 型レクチンである DC-SIGN（Dendritic cell intracellular adhesion molecule 3-grabbing non-integrin）、 DC-SIGNR（Dendritic cell intracellular adhesion molecule 3-grabbing non-integrin-related protein）、ASGPR（asialoglycoprotein receptor）および MGL（macrophage galactose-type C-type lectin）によって１０倍から１００倍ほど増強される。C 型レクチンは本来糖鎖を認識して病原体の排除等に関わる分子であるため、C 型レクチ

ンによる感染性西ナイルウイルス様粒子の感染増強に西ナイルウイルスE蛋白質の糖鎖が関わることが予想される。E蛋白質の154番目のアミノ酸アスパラギン（N154）はN型糖鎖修飾を受けるので、このアミノ酸をアラニンにしてN型糖鎖修飾を受けないようにしたE蛋白質変異体（N154A）を作製し、野生型のものと同様に感染性西ナイルウイルス様粒子を作製した。N154A変異体のウイルス様粒子はC型レクチンを発現していないJurkat細胞では野生型ウイルス様粒子と同程度の感染性を示した。このことはN154Aの変異がウイルス様粒子の形成性やC型レクチンを介さない感染には影響しないことを示すと考えられる。しかしC型レクチン発現細胞への感染は、N154A変異体ウイルス様粒子の場合には全く増強されなかった。西ナイルウイルスE蛋白質の154番目アミノ酸の糖鎖付加がC型レクチンによる感染増強に関わることが示された。

2.　組換えインフルエンザウイルスの作製

インフルエンザウイルス（感染昆虫細胞が蛍光蛋白質を発現するようになるもの）の低温馴化にあたり、まず低温馴化株FluMistのウイルス各遺伝子を用いた方が後の低温馴化が行いやすいと考えた。そこでVSVG(HA)（インフルエンザウイルスWSN株のHA分節にVSVG遺伝子を入れたもの）とVenus(NA)（WSN株NA分節にVenus）以外の6分節をすべてFluMist由来のものにしてリバースジェネティクスを行ったが、増殖性のあるウイルスは得られなかった。そこで5分節をWSN株由来、1つのみFluMist由来としてウイルス回収を試みたところ、PB1, NP, PA, NS分節をFluMist由来にした場合に増殖性のウイルスを得ることができた。M, PB2分節をFluMistにした場合には増殖性ウイルスは得られなかった。その他いくつかの組み合わせで検討を行なったところ、8種の分節組み合わせで増殖性のあるウイルスが得られた。

(4) 等温遺伝子増幅産物の検出に向けたイムノクロマト・ストリップ作成法の確立

1．　FITC-Biotin標識*B. gibsoni* P50遺伝子増幅産物を検出のための最適なイムノクロマト・ストリップの作製

ニトロセルロースメンブレンHF180MC100, ストレプトアビジン標識金コロイド粒子をコンジュゲートパットに浸漬させ乾燥させたコンジュゲートパット(No.8964：ミリポア）を用いて、イムノクロマト・ストリップを作製した。作製したイムノクロマト・ストリップのSample application padに1で増幅させたFITC-とBiotin-でラベルした*B. gibsoni* P50遺伝子のPCR増幅産物を用いて評価した。その結果、原虫感染の明瞭な検出バンドが認められた（Detection band）、しかしながら、コントロールバンドには認められなかった。イムノクロマト・ストリップを半年間室温に保存し、PCRにより得られた*B. gibsoni* P50遺伝子増幅産物を用いて検討を行った。その結果、半年前と同様の検出バンドが確認された。以上の結果より、本イムノクロマト・ストリップにおける*B. gibsoni*の検出は少なくとも半年は使用可能であることを確認した。

(5) ネズミマラリア原虫を用いたヒトマラリア原虫感染モデルの構築

1. ネズミマラリア原虫テロメア配列のクローニング

PCRにてネズミマラリア原虫ゲノムよりテロメアのクローニングを試みたところ200-300bpのDNA断片が増幅された。増幅した断片をプラスミドにクローニングし配列を確認した。その結果、Ponzi M.らと同様の5’-TT(T/C)AGGG-3’配列が反復した約200bpのテロメア配列をクローニングすることに成功した。これを用いて人工染色体構築を試みた。構築した人工染色体の概略図を示す。最終的に得られる直鎖状の人工染色体は(Linear Plasmodium Artificial chromosome：L-PAC)と名づけた。

2. ネズミマラリア原虫血液感染ステージへの人工染色体の導入と安定性の評価

構築したPACをネズミマラリア原虫へと導入した。その結果、導入後の寄生率（全赤血球に対する感染赤血球の割合（%））の上昇は明らかにL-PACの方が環状人工染色体やコントロールプラスミドより速いことが示された。寄生率の差から導入効率を予測し、比較すると、L-PACは環状人工染色体と比べ、約100倍、コントロールプラスミドとは約1000倍以上、遺伝子導入効率が上昇した結果となった。

3. ベクター感染ステージでの導入染色体の安定性の評価

続いてPACの原虫内での安定性を評価した。その結果、PACは環状人工染色体と同程度の安定性を持つことが明らかとなり、テロメアの付加と直鎖化が人工染色体の分配に影響を与えないことが示された。蚊ステージについ