Molecular interactions between *Anopheles stephensi* midgut cells and *Plasmodium berghei*: the time bomb theory of ookinete invasion of mosquitoes

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We present a detailed analysis of the interactions between *Anopheles stephensi* midgut epithelial cells and *Plasmodium berghei* ookinetes during invasion of the mosquito by the parasite. In this mosquito, *P.berghei* ookinetes invade polarized columnar epithelial cells with microvilli, which do not express high levels of vesicular ATPase. The invaded cells are damaged, protrude towards the midgut lumen and suffer other characteristic changes, including induction of nitric oxide synthase (NOS) expression, a substantial loss of microvilli and genomic DNA fragmentation. Our results indicate that the parasite inflicts extensive damage leading to subsequent death of the invaded cell. Ookinetes were found to be remarkably plastic, to secrete a subtilisin-like serine protease and the GPI-anchored surface protein Pbs21 into the cytoplasm of invaded cells, and to be capable of extensive lateral movement between cells. The epithelial damage inflicted is repaired efficiently by an actin purse-string-mediated restitution mechanism, which allows the epithelium to 'bud off' the damaged cells without losing its integrity. A new model, the time bomb theory of ookinete invasion, is proposed and its implications are discussed.

Keywords: actin/malaria/nitric oxide synthase/subtilisin/wound repair

Introduction

Malaria infections impose a tremendous burden to human health in tropical regions worldwide, particularly in Africa. Although anopheline mosquitoes play a central role in transmission of the malaria parasite, *Plasmodium*, our understanding of the basic biology of vector–parasite interactions during the transit of the parasite through the mosquito is still limited. Upon ingestion, *Plasmodium* gametocytes differentiate into gametes within the midgut lumen, fertilization takes place and the zygote develops into an ookinete, which penetrates the midgut epithelium and forms an oocyst on the basal side. When the oocyst matures and ruptures, sporozoites are released into the haemolymph and invade the salivary glands, to be injected into a new host when the infected mosquito feeds again.

Malaria transmission is possible because some parasites are able to develop and escape unharmed from the mosquito.

There is increasing evidence that the mosquito midgut is an immune-competent organ, inducing the expression of several immune markers in response to *Plasmodium* infection. In *Anopheles gambiae* (Ag), mRNA levels of the antibacterial peptide defensin, a putative Gram-negative binding protein (AgGNBP), a chitinase-like domain-containing protein (ICHIT), a putative serine protease (ISPL5), a lectin-like protein (IGALE20) and nitric oxide synthase (AgNOS) increase in the midgut 24 h post-infection with *Plasmodium berghei* (Dimopoulos *et al.*, 1997, 1998; Richman *et al.*, 1997; reviewed by Barillas-Mury *et al.*, 2000). Studies in *Anopheles stephensi* (As) have also shown transcriptional activation of AsNOS in the midgut and systemically in response to *P.berghei* infection. Furthermore, dietary provision of the NOS substrate L-arginine significantly reduces the percentage of *Plasmodium*-infected mosquitoes, while an NOS inhibitor significantly increases the number of oocysts that develop (Luckhart *et al.*, 1998). These immune responses have been observed in mosquito strains of *A.gambiae* and *A.stephensi* susceptible to *P.berghei* infection.

Previous studies of *Aedes aegypti* midgut invasion by the chicken malaria parasite *Plasmodium gallinaceum* reported that ookinetes invade a special type of epithelial cells expressing high levels of vesicular ATPase (vATPase), which were named 'Ross cells'. The histochemical and electron microscopic analysis of the morphology of these cells indicated that they differ from other epithelial cells. They stain very poorly with toluidine blue (a basophilic dye), are less osmophilic, contain minimal endoplasmic reticulum, lack secretory granules and have few microvilli (Shahabuddin and Pimenta, 1998). The same group has also reported that *P.gallinaceum* ookinetes invade a similar type of cell in *A.gambiae* midgut cells expressing high levels of vATPase (Cociancich *et al.*, 1999). The conclusion that *P.gallinaceum* ookinetes do invade functionally related cells in both *A.aegypti* and *A.gambiae* was drawn from very few invasion events, due to the use of an unfavourable insect–parasite combination.

The existence of 'Ross cells' has important implications. If ookinetes have developed a mechanism to invade a specific midgut cell type, this implies the existence of a recognition system. One could envisage that interactions between ookinetes and specific receptors, presumably present only on the surface of the 'Ross cells', are required for successful midgut invasion, and thus would represent potential target molecules to block disease transmission.

In this study, the cell biology of the interactions between midgut cells and ookinetes during epithelial invasion are examined in the well-established *A.stephensi*–*P.berghei* malaria model, in which numerous ookinetes succeed in

developing into oocysts. Conclusions are drawn after observing hundreds of invasion events in this compatible mosquito–parasite combination, where manipulations of the midgut tissue were minimized to prevent *in vitro* cell damage. Ookinetes were localized using monoclonal antibodies to Pbs21, an abundant surface protein, and their interactions with midgut epithelial cells were established by analysing the expression of several proteins, such as AsSTAT, vATPase, AsNOS, actin and a *P.berghei* subtilisin-like serine protease, PbSub2. The data show that, in *A.stephensi* mosquitoes, *P.berghei* ookinetes invade columnar midgut epithelial cells with microvilli, which are capable of mounting anti-parasitic responses, such as the induction of NOS expression, and that parasite invasion always results in cell death. *Plasmodium berghei* ookinetes do not invade the subset of cells expressing high levels of vATPase. Invaded cells are functionally and morphologically different from healthy midgut epithelial cells, but these differences are the consequence of the damage inflicted by the parasite and are not present before invasion. A new model of ookinete invasion, the time bomb theory, is proposed.

Results

Midgut epithelial cells invaded by ookinetes fail to translocate AsSTAT

Two transcription factors, Gambif1 (gambiae immune factor 1), which belongs to the rel-family (Barillas-Mury *et al.*, 1996), and AgSTAT, a member of the STAT family of receptors, have been characterized previously (Barillas-Mury *et al.*, 1999). Both factors are activated in fat body cells following a bacterial challenge and are also expressed in the midgut epithelial cells of *A.gambiae* (Barillas-Mury *et al.*, 1996, 1999). Pervanadate treatment (hydrogen peroxide and vanadate) is known to activate the STAT pathway in vertebrates and *Drosophila* through a ligand-independent mechanism (Zhong *et al.*, 1994; Yan *et al.*, 1996). AgSTAT is evenly distributed in the cytoplasm and nucleus of midgut cells, but pervanadate treatment results in a prominent translocation of AgSTAT from the cytoplasm to the nucleus, indicating that this pathway has been activated (Barillas-Mury *et al.*, 1999). A polyclonal antiserum was generated previously using a region of AgSTAT with 54% amino acid identity (80% similarity) to the *A.stephensi* homologue, AsSTAT (C.Barillas-Mury, unpublished). The anti-AgSTAT antibody recognizes a single band of 78 kDa in adult *A.stephensi* female extracts (data not shown), with the same pattern as the immunoblot that has been reported for *A.gambiae* extracts (Barillas-Mury *et al.*, 1999). This antibody also cross-reacts with *A.stephensi* midgut tissue in immunostainings and was used to determine the subcellular distribution of AsSTAT in *P.berghei*-infected midguts.

The location of *P.berghei* ookinetes was determined using a monoclonal anti-Pbs21 antibody (Winger *et al.*, 1988). Pbs21 is a surface protein expressed at high levels in *P.berghei* zygotes and ookinetes; it is present at lower levels in oocysts and absent in sporozoites and blood-stage parasites (Simonetti *et al.*, 1993). Pbs21 has four epidermal growth factor-like domains, a putative C-terminal hydrophobic membrane anchor and sequence homology

Fig. 1. Immunofluorescence staining of midguts with anti-Pbs21 (green) and anti-STAT (red) antibodies, 8 h after feeding on an ookinete culture. (A) Some cells protrude to the luminal side and their cytoplasm stains with Pbs21. (B) An ookinete emerging from a Pbs21-positive cell. The invaded cell has very low AsSTAT expression and the nucleus does not stain. (C) Pbs21-positive cells fail to translocate AsSTAT, even after pervanadate treatment. Their location in the red channel is indicated by the arrows. The small arrowheads indicate some examples of nuclei with strong AsSTAT staining.

(45, 45 and 40%, respectively) to 25 kDa surface proteins found in *P.falciparum* (Pfs25), *P.reichenowii* (Prs25) and *P.gallinaceum* (Pgs25) (Paton *et al.*, 1993). Biochemical analysis indicates that Pbs21 is glycosylated and GPI anchored (Blanco *et al.*, 1999).

Double staining for AsSTAT and Pbs21 in midguts from females 8 h after feeding on ookinete cultures revealed that Pbs21 is present throughout the cytoplasm of some midgut epithelial cells (Figure 1A). In many cases, intact ookinetes could be observed emerging from cells with moderate Pbs21 cytoplasmic staining. Most probably this staining is due to secretion of this surface protein during the parasite's transit across the cell (Figure 1B). A small area rich in Pbs21 was often observed on the luminal surface of invaded cells, and could represent the site of parasite penetration. Arrowheads in Figures 1B and 2G indicate examples of such areas. The Pbs21 staining in some cells was very strong, although ookinetes could not be observed in the immediate vicinity; we surmised that in these cases the parasite may have been lysed intracellularly. AsSTAT was present in the cytoplasm and nucleus of most epithelial cells. However, all cells positive for Pbs21 were notable for having only a very low cytoplasmic level and lack of nuclear staining for AsSTAT (Figure 1B). To determine whether Pbs21-positive cells were capable of translocating AsSTAT, ookinete-infected midguts were treated *in vitro* with pervanadate before fixation. As expected, this treatment resulted in a dramatic redistribution of AsSTAT from the cytoplasm to the nucleus in most midgut cells. Again, however, AsSTAT staining was very weak in all Pbs21-positive cells, and was absent from the nucleus (Figure 1C).

These results indicate that the ookinete-invaded cells are functionally different from other midgut epithelial cells. Two possible explanations exist. The invaded cells could belong to a special cell type that is targeted specifically by ookinetes, unable to activate the AsSTAT pathway and equivalent to the 'Ross cells' described for *A.egypti*. Alternatively, inability to activate the AsSTAT pathway may be the result of cell damage inflicted by the parasite during invasion. To test these hypotheses, a detailed analysis of the cellular morphology and the expression and distribution of several protein markers was performed.

The invaded cells have a distinct morphology and overexpress NOS

In all subsequent experiments, unless stated otherwise, midgut tissues were analysed 24 h after feeding either on naive or malaria-infected mice. Single or multiple immunofluorescence staining was detected by confocal microscopy in multiple optical sections, which were then merged to visualize the various markers at different planes of the midgut tissue. In an initial study, double immunofluorescence staining for AsNOS and Pbs21 was performed. AsNOS was detected using a commercial 'Universal anti-NOS' antibody (Oncogene, Cambridge, MA), raised against a conserved region of mouse iNOS that is known to cross-react with multiple species. In immunoblots, the antibody specifically detected a single band of the expected size (150 kDa) in *A.gambiae* and *A.stephensi* extracts from unfed females and pupae (data not shown).

AsNOS is constitutively expressed in the cytoplasm of most midgut epithelial cells fed on normal blood. Morphological examination using differential interference contrast (DIC) revealed a smooth epithelium, in which epithelial cells of regular shape are present in a single plane, in a symmetrical distribution (Figure 2A). In contrast, following ookinete invasion, several cells were observed by DIC that protrude to the luminal side of the midgut, express very high levels of NOS and are closely associated with Pbs21-positive ookinetes (Figure 2A). The protruding cells are never observed in uninfected controls, suggesting that the morphological differences and very high NOS levels observed following ookinete invasion are the result of damage caused by the parasite. In ~95% of the cases, healthy parasites that had reached the basal lamina co-localized with cells that protruded to the lumen and overexpressed NOS at 24 h post-feeding (Figure 2A and F). Damaged cells without associated parasites and healthy parasites (Figure 2H), not in contact with damaged cells, are also observed.

Plasmodium berghei does not invade a subpopulation of cells expressing high levels of vATPase in *A.stephensi*

To determine whether *P.berghei* ookinetes in *A.stephensi* invade the functional equivalent of the *A.aegypti* vATPase-expressing 'Ross cells' (Shahabuddin and Pimenta 1998), the expression of AsNOS and vATPase in ookinete-invaded cells was analysed in parallel. A rat polyclonal antiserum to the vATPase F subunit from *A.gambiae* was generated, as the *A.aegypti* anti-vATPase antiserum, kindly provided by Dr Shahabuddin, did not

Fig. 2. Immunofluorescence staining of midguts with anti-NOS, anti-vATPase and anti-Pbs21. (A) DIC, NOS and Pbs21 staining of midguts 24 h after feeding on a healthy (control) or malaria-infected mouse. (B) Western blot of adult female homogenates from *A.gambiae* and *A.stephensi* stained with pre- or post-immune serum to Ag-vATPase. (C) Double staining of NOS (green) and vATPase (red). The cells expressing high levels of vATPase do not express NOS. The inset illustrates the NOS staining channel of a columnar cell and a small vATPase-overexpressing cell (indicated by the arrow) at higher magnification. The arrowheads indicate the nuclei; note the size difference between the two cell types and the lack of NOS staining in the small cell. (D–G) Triple stainings of infected midguts for Pbs21 (red), NOS (green) and vATPase (blue). (D) Homogeneous AsNOS cytoplasmic staining of epithelial cells at the posterior end of the midgut. (E) Small cells expressing high levels of vATPase were observed in the same sample as in (D); one is indicated by the arrow. (F) Ookinetes co-localize with protruding cells expressing high levels of NOS. (G) The protruding cells express the same level of vATPase as the uninvaded cells. (H) An ookinete that has reached the basal lamina is in close proximity to a vATPase-overexpressing cell.

cross-react with *A.stephensi* in immunoblots or immunofluorescence staining (data not shown). In immunoblots of *A.gambiae* and *A.stephensi* whole female extracts, the anti-vATPase F rat antiserum detected a single band of the predicted size (~13 kDa), which was not detected by the pre-immune serum of the same animal (Figure 2B).

NOS and vATPase double stainings were performed in midguts of mosquitoes fed normal mouse blood. The anti-Ag-vATPase antibodies detected a subpopulation of cells that overexpress vATPase, but do not express AsNOS. These cells are small, flat, have a triangular shape, are only seen on the basal side of the epithelium and are more abundant in the posterior end of the midgut (Figure 2C). These morphological features make it hard to envisage how they could be successfully invaded by ookinetes.

To detect protruding cells, ookinetes and the cells overexpressing vATPase simultaneously, triple staining for Pbs21, NOS and vATPase was performed. The posterior region, where the cells overexpressing vATPase are more abundant, was analysed initially to ensure that these cells were stained properly. Constitutive cytoplasmic expression of AsNOS in columnar epithelial cells (Figure 2D) and high levels of vATPase in the

small triangular cells were observed (Figure 2E). Subsequently, images were obtained from a different area of the same midgut sample where ookinetes were present. Multiple cells, expressing high levels of AsNOS and associated with ookinetes, were observed protruding into the midgut lumen. The protruding cells were spherical, appeared to be about to 'bud-off' from the epithelium and remained attached only by a narrow area at the base (Figure 2F). The protruding cells stain only weakly for vATPase, with a similar intensity to that of uninvaded cells (Figure 2G).

After scanning hundreds of ookinetes, it was possible to find only one that had reached the basal lamina and was in the same field of view and plane as a nearby cell

overexpressing vATPase (Figure 2H). A cell protruding to the lumen was, however, also observed in close proximity by DIC (data not shown). The lack of co-localization of vATPase-overexpressing cells with ookinetes, therefore, again indicates that these are not the targets for ookinete invasion.

Ookinete invasion results in extensive cell death

To determine whether the protruding cells were irreversibly damaged, the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labelling (TUNEL) DNA fragmentation assay was performed. In this assay, terminal transferase enzyme is used to incorporate fluorescence-labelled dNTPs at the free ends of genomic DNA. The efficiency of label incorporation depends on the number of free ends in the DNA strand. When the cell has been irreversibly damaged, DNA fragmentation generates numerous free ends, which result in strong fluorescence staining. Midgut cells that were damaged by a longitudinal incision made to remove the blood meal contents and create a flat midgut epithelial sheet provided a positive control. In the interior of the sheet, all ookinete-invaded cells protruded to the lumen and their nuclei were strongly TUNEL positive (Figure 3A). We have also observed in such preparations that all protruding cells have lower levels of AsSTAT expression and lack nuclear staining (data not shown).

While the evidence for DNA fragmentation in invaded cells is clear, it is uncertain whether this is indicative of programmed cell death (apoptosis). DNA laddering tests of midgut DNA followed by a species-specific hybridization revealed nucleosome-like ladders indicative of apoptosis in the cells of the mouse blood meal, but not in the mosquito midgut cells, irrespective of the presence or absence of infective ookinetes (data not shown). Most probably, the amount of fragmented DNA from the damaged midgut cells is below the limit of detection of this assay. Thus, further analysis would be required to establish whether ookinetes are actively inducing apoptosis of the invaded cells. Gross changes in nuclear morphology were also often observed in protruding cells (Figure 3B). Whilst the nuclei of normal cells are circular, symmetrical and show homogeneous DNA distribution, those of invaded

Fig. 3. (A and B) DIC imaging, TUNEL assay (green) and DAPI nuclear staining (blue) of midgut epithelial cells 24 h post-feeding on malaria-infected mice. The specimen in (B) is the same as that in (A), at higher magnification, comparing the nucleus of a healthy cell with that of an invaded cell that protruded to the lumen. (C) Staining of protruding cells for NOS (red) and actin (Alexa-488 conjugated phalloidin in green). The invaded cells present actin aggregates and a loss of surface microvilli in the midgut lumen side (ml). Compare the microvilli density on the surface of an invaded cell (white box in the ml plane) with that of a healthy cell (white box in the bl plane). An actin ring is formed at the base of the cell, in the plane of the basal lamina (bl). The arrows indicate the direction of the actin ring constriction that would be required to bud off the cell into the midgut lumen. (D) Double staining of an invaded cell for actin (green) and Pbs21 (red). Notice the loss of microvilli (compare white boxes), the actin aggregates (indicated by the arrowheads), the constriction of the parasite at the point where it emerges from the cell and the actin ring at the base of the cell. (E) Lateral view of the parasite-invaded cell in (D). (F) Midgut staining 48 h after infection for NOS (green) and Pbs21 (red). Early oocysts can be observed in a healthy looking midgut that has been repaired.

cells appear pycnotic, with irregular shapes. They present areas of DNA condensation and, in some cases, partial fragmentation of the nucleus.

Dead cells 'bud off' into the midgut lumen as the epithelial damage is repaired through an actin purse-string-mediated restitution mechanism

During experimental *P.berghei* infections in *A.stephensi*, it is not uncommon to observe >100 successful ookinete invasions within a single midgut. Our results indicate that all invaded cells die and that a single parasite often injures more than one cell. Furthermore, ookinete invasion causes this potentially massive damage at a time when the midgut is distended by the blood meal and high levels of proteases are present in the lumen. However, no difference in mortality between females fed on uninfected or infected mice is observed, with >96% of infected females surviving 48 h after infection. How does the midgut manage to repair such extensive damage, preserve the continuity of the epithelium and thus prevent direct contact between the haemolymph and the midgut contents? Disruptions of the mucosal lining of the gastrointestinal tract of vertebrates are resealed by the concerted movement of the surrounding cells, a process termed restitution, and not by cell division (Lotz *et al.*, 2000). Epithelial sheets respond to injury by mobilizing their actin cytoskeleton through two different mechanisms: purse-string contraction and formation of lamellae. A detailed analysis of the actin cytoskeleton, using phalloidin conjugated to a fluorescent dye, was performed to determine the type of healing mechanism that takes place in the mosquito midgut epithelium.

A dramatic redistribution of actin from the luminal to the basal side of the invaded midgut cells was observed (Figure 3C and D). Serial sections were taken across the epithelium from apical to basal, and were separated into two stacks before merging: the more apical stack including the midgut lumen (ml) and the more basal one the basal lamina (bl). In Figure 3C, panel ml visualized only protruding cells, which are NOS rich and depleted of microvilli (white box), except at their periphery. Panel bl includes the microvillar apical region of non-invaded cells (white box) as well as the more basal region of the invaded cells, which shows actin aggregates forming ring structures. In Figure 3D, panel ml, the two white boxes permit direct comparison of microvilli-rich non-invaded cells (peripheral) and a microvilli-poor invaded cell (central), in which the actin is aggregated around a Pbs21-stained parasite; panel bl clearly shows the actin ring. We propose that such rings are responsible for constricting the protruding cells at their base, allowing the epithelium eventually to pinch these cells off and expel them into the midgut lumen, whilst simultaneously closing the gap they would otherwise leave behind (Figure 5C). When several contiguous cells are damaged, they are expelled from the epithelium as a group (Figures 2F and 3C).

A three-dimensional image of the invaded cell in Figure 3D was reconstructed using all sections and rotated to allow a lateral view of the same cell (Figure 3E). The cell protrudes towards the luminal side, the parasite itself is constricted by the actin at the basal surface of the cell and the adjacent cell has flattened at the site of contact with the injured cell. Parasite constrictions are seldom observed

(see below); they could be either rare events or very transient ones. The epithelial repair mechanism is very effective; by 48 h after feeding, most of the damaged cells have been expelled to the lumen and early oocysts are observed developing in a healthy looking midgut (Figure 3F).

***P.berghei* ookinetes are remarkably plastic and are capable of extensive lateral movement between cells**

A novel ookinete morphology has been described recently for *P.gallinaceum*, in which the central portion of the parasite forms an elongated narrow tube or stalk joining the anterior and posterior portions of the parasite. Stalk-form ookinetes were not observed *in vitro*, and thus it was postulated that they result from interactions of the parasite with the midgut epithelium (Vernick *et al.*, 1999). Hundreds of Pbs21-stained parasites were examined to determine whether the stalk morphology also exists in *P.berghei* ookinetes as they traverse the *A.stephensi* midgut epithelium. More than 99% had the typical 'banana shape' (Figure 4A). Several examples of variations of the stalk shape were found, however, ranging all the way from moderate constrictions in the posterior end of the parasite (Figure 4B) to dramatic elongated stalks in the middle (Figure 4C).

Electron microscopic analysis of a stalk-shaped *P.gallinaceum* ookinete crossing laterally between two cells revealed that the point of entry of the stalk into the adjacent cell is surrounded by a halo of fine fibrillar material that resembles actin filaments (Vernick *et al.*, 1999). To determine whether the stalk morphology correlated with interactions between ookinetes and the midgut actin cytoskeleton, double staining for Pbs21 and actin was performed. The stalk-shaped ookinetes were always found in the process of emerging from the basal side of the invaded midgut cell, with the stalk region surrounded by actin aggregates (Figure 4E and F). In two cases, the stalk region was torn apart, bisecting the parasite (Figure 4D). The stalk region, therefore, appears to be generated as the parasite pushes forward to escape from the midgut cell, while being locally constrained by actin aggregates.

Occasionally, we observed several adjacent cells that were damaged and a single parasite that was emerging from the last cell, suggesting that the same parasite crossed several cells by lateral invasion. Furthermore, examples of parasites that left clearly visible Pbs21 trails as they moved across several contiguous cells were found (Figure 4G). At the extreme, an ookinete was observed that had moved extensively in the lateral direction, leaving behind a row of six 'bald' damaged cells with very few microvilli (Figure 4H).

Ookinetes release a subtilisin-like serine protease, PbSub2, during mosquito midgut epithelial invasion

Two subtilisin-like serine proteases, PfSub1 and 2, are expressed in merozoites, the invasive form of the malaria parasite during blood-stage development (Blackman *et al.*, 1998; Barale *et al.*, 1999; Hackett *et al.*, 1999; Sajid *et al.*, 2000). These and other protease activities have been implicated in the invasion process, as potential mediators

Fig. 4. (A–D) Immunofluorescence staining of ookinetes with Pbs21 (red) and detection of haemozoin granules (yellow). (E and F) Double staining for Pbs21 (red) and actin (green). Note the close association of actin with the parasite constrictions (indicated by the arrows). (G) Immunofluorescence staining of cells following ookinete invasion with anti-NOS (red) and anti-Pbs21 antibodies (green), counterstained with DAPI (blue). The area where the Pbs21 trail is present was overexposed, as the signal was much weaker relative to that of the ookinete surface. (H) Double staining for actin (green) and Pbs21 (red). The same ookinete has undergone extensive lateral movement, damaging six consecutive cells. (I) Immunoblot of cultures containing schizonts and trophozoites with recombinant PbSub2 pre- and post-immune serum. (J and K) Triple staining for actin (green), Pbs21 (light blue) and subtilisin (PbSub2 in red). The arrows in (J) indicate the close contact between the PbSub2 aggregates and the actin cytoskeleton. All aggregates (including the one indicated by the arrow in K) are present in the cytoplasm of the invaded cell, and are presumably formed after PbSub2 is secreted by the parasite.

for processing the merozoite surface protein 1 (MSP1), and modifying the red blood cell surface (Blackman and Holder, 1992; Cooper and Bujard, 1992; McPherson *et al.*, 1993; Roggwiler *et al.*, 1996). We have identified the orthologue of PfSub2 in *P. berghei*, PbSub2 (63% identity, 80% similarity within the catalytic domain). A polyclonal antiserum against recombinant PbSub2 protein specifically detects by immunoblotting two major processed PbSub2 fragments of 64 and 58 kDa in *P. berghei* cultures

Fig. 5. Diagrammatic representation of the time bomb model of ookinete invasion. (A) Parasite invasion results in induction of NOS expression, the release of NO and damage to the invaded cell. (B) Ookinetes are capable of lateral movements from one cell to another, resulting in damage of adjacent cells. (C) The invaded cells are damaged irreversibly and are extruded from the epithelium by a constricting actin ring at the base of the cell. This mechanism allows the midgut to repair the damage, without losing the continuity of the epithelium. Ookinetes are capable of extensive lateral movement in the space between the epithelial cells and the basal lamina, before they differentiate into oocysts.

containing schizonts and trophozoites (Figure 4I). A minor species of 130 kDa, probably corresponding to the PbSub2 precursor protein, is also detected at low levels when large amounts of extract are used (data not shown).

This pattern is in agreement with that reported for *P.falciparum* subtilisin-2 (Hackett *et al.*, 1999).

To investigate whether ookinetes express and therefore may utilize subtilisin activity to move through the midgut cells, we have used the anti-PbSub2 antibody to localize PbSub2 during parasite invasion of the mosquito midgut. Triple immunofluorescence staining for Pbs21, PbSub2 and actin revealed that PbSub2 is secreted into the cytoplasm of ookinete-invaded cells, forming protein aggregates (Figure 4J and K) that are often associated with the actin cytoskeleton (arrows in Figure 4J). PbSub2 stains the cytoplasm of the ookinete with a granular pattern. Careful examination of individual confocal sections revealed that all large aggregates (including the one indicated by the arrow in Figure 4K) are found in the cytoplasm of the invaded cell, and are presumably formed after the protease is secreted by the parasite. PbSub2 staining was not observed in adjacent healthy cells, in uninfected females or after staining with pre-immune serum (data not shown). Thus, PbSub2 is expressed by the parasite during invasion in both the vertebrate and mosquito host, and is secreted into the invaded mosquito midgut cells, where potentially it may modify the cytoskeletal network.

Discussion

The time bomb theory of ookinete invasion

A combination of morphological analysis and immunostaining for molecular markers has clearly established that *P.berghei* ookinetes do not invade the limited number of *A.stephensi* midgut cells that stain strongly for vATPase. Instead, the parasites apparently enter normal, low vATPase midgut columnar epithelial cells that, when invaded, undergo a series of morphological and molecular changes reflecting irreversible damage that ultimately leads to cell death. These cells protrude into the midgut lumen, express high levels of NOS, acquire abnormal nuclear morphology, fragment their DNA and radically reorganize their actin cytoskeleton. They deplete their apical region of microvilli while forming an actin ring near their basal region. Later constriction of the ring may result in the extrusion of these cells from the epithelium, as the epithelium has been restored to its previous normal appearance by 48 h post-infection (Figure 5).

As the parasite invades a cell, not only does it inflict physical damage, but it also secretes substances such as the GPI-anchored surface protein Pbs21 and the PbSub2 protease, which might facilitate the invasion process. Parasite invasion activates a series of defence responses, such as NOS production, and initiates pathways that ultimately lead to cell death, thus triggering a time bomb (Figure 5A). The abundance of NOS and blood-derived arginine at this time suggests that large amounts of nitric oxide (NO) may be generated. In this scenario, the relationship between the speed at which the parasite crosses the cell(s), the rate of toxic NO production and how long the cell can sustain this and other possible defence responses, as it undergoes cell death, would be critical to determine the outcome of invasion. Ookinetes would have a limited time window to emerge unharmed from the cell, and the observed extensive lateral movement could be a strategy to migrate from the luminal to the basal

side, while moving away from the source of toxic chemicals, such as NO (Figure 5B).

We were surprised to find that, at the level of epithelial invasion, the battle seems remarkably one sided in favour of the parasite. The invaded cells induce NOS expression, but the parasites appear to escape unharmed in at least 95% of the cases where we observed co-localization of a healthy looking parasite with damaged cells. Our results may indicate that the remarkable ability of parasites to move across the invaded cells allows them to escape the defence responses, while leaving behind a single or sometimes a trail of doomed epithelial cells. In general, only a small percentage of mature ookinetes are successful in reaching the basal lamina and forming oocysts (reviewed by Ghosh *et al.*, 2000). Our data would suggest that the major losses happen before the parasites reach the cytoplasm of the invaded cell and/or after oocyst formation. As NO is very toxic and highly diffusible, the induction of NOS could have dramatic effects on the ookinetes present in the lumen of the midgut or early oocysts.

The evidence is clear that ookinete invasion results in characteristic changes in midgut epithelial cell morphology and cytoskeletal organization. The damaged columnar cells protrude to the lumen and exhibit a dramatic loss of microvilli, while a constricting purse-string actin ring is generated on the basal side of the cell (Figure 5C). The damage is extensive and irreversible, resulting in cell death. As the actin ring contracts, the dead cells 'bud off' the midgut epithelium, and the adjacent cells flatten to increase their surface area and cover the space left by the extruded cells.

A parallel exists in the interaction of *Plasmodium* with cells of the vertebrate host. Sporozoites are capable of inducing cell death in macrophages (Danforth *et al.*, 1980). The parasite also releases circumsporozoite (CS) surface protein into the cytoplasm of invaded hepatocytes, which associates with ribosomes and blocks translation (Hugel *et al.*, 1996; Frevert *et al.*, 1998).

An important caveat to the picture of a one-sided battle is that *A.stephensi* is highly susceptible to *P.berghei*, while *A.gambiae* is less hospitable to this parasite. It would be interesting to compare the cell biology of invasion in these two mosquitoes, at the morphological and molecular levels.

Epithelial healing in response to injury

The model that we propose resembles the classic purse-string epithelial closure mechanism that has been established in both vertebrates and flies. The leading edge of the lateral epidermis of *Drosophila* embryos also forms a contractile purse-string that provides the traction required for dorsal closure, and the same wound healing mechanism is triggered by the epidermis in response to mechanical and UV laser-induced injury (Kiehart *et al.*, 2000). During epithelial wound healing by purse-string contraction in vertebrates, the cells at the margin of the damaged area reorganize their actin cytoskeleton, forming a ring that contracts to close the injury (Lotz *et al.*, 2000). Lamellae are large, flat cytoplasmic protrusions that are extended by the marginal cells into the denuded area and are often used to repair large wounds (Lotz *et al.*, 2000). Studies of fibroblast motility indicate that the actin cytoskeleton

associates with integrins on the lamellar surface, and that the traction to pull the cell forward is generated by the interaction between integrins and their extracellular matrix ligands. Following adhesion, the lamellae contract, allowing the cell to translocate (Sheetz *et al.*, 1998). The repair observed in the mosquito midgut epithelium differs from the classic purse-string model, in that the damaged cell itself (rather than the surrounding cells) forms the constrictive actin ring.

How universal is this invasion model?

The relevance of the time bomb theory to human health is determined by the extent to which *P.berghei*, the murine malaria parasite, is a valid model for human malaria transmission by its natural vectors *A.gambiae* and *A.stephensi*. This question is more critical now that *P.berghei* and *A.stephensi* are both amenable to genetic manipulation (Waters *et al.*, 1997; Catteruccia *et al.*, 2000), making this a very powerful model system for development of new experimental and control strategies.

A comparative analysis of the induction of NOS activity, using diaphorase staining in *A.stephensi* following infection with *P.berghei* and *P.falciparum* (Luckhart *et al.*, 1998), revealed that 24 h after feeding both species induce NOS activity in individual cells concentrated in the posterior midgut, the region where *Plasmodium* development typically occurs. A weak uniform staining was observed in uninfected midguts. Furthermore, double stainings with diaphorase and propidium iodide demonstrated that, in some cases, the immature oocysts of both species co-localized with the positive cells. Administration of the NOS inhibitor *N*^G-nitro-L-arginine methyl ester (L-NAME) to *P.falciparum*-infected females resulted in an almost 2-fold increase in the number of developing oocysts (Luckhart *et al.*, 1998). In agreement with these results, previous electron microscopic (EM) studies indicate that, in *A.stephensi* 24 h post-infection, *P.berghei* takes an intracellular route, resulting in cell damage (Meis *et al.*, 1989). *Plasmodium falciparum* ookinetes analysed 29–34 h post-infection, however, were found to cross the epithelium through the intercellular space between healthy looking epithelial cells in the same mosquito species (Meis *et al.*, 1989). Interestingly, in these studies, the intercellular ookinetes were observed exclusively in very close proximity to the basal lamina. Since the EM studies on *P.falciparum* invasion were performed between 29 and 34 h after feeding, rather than 24 h as in the *P.berghei* study, a possible explanation is that *P.falciparum* ookinetes do invade the midgut through an intracellular route, at ~24 h, but 5–10 h later they have moved away from the initial invasion site.

Contrary to the widespread idea that ookinetes arrest their movement as soon as they come in contact with the basal lamina, we have observed that 24 h after feeding, after reaching the space between the epithelial cell and the basal lamina, ~5% of the ookinetes have moved away from the nearest invaded cell by as much as 5–6 cell diameters (see Figure 5C). Moving away from an invasion site, where highly toxic and diffusible NO is generated, would be beneficial to the parasite. In agreement with this proposed hypothesis, *P.gallinaceum* ookinetes were observed in both intra- and intercellular positions in the

midgut epithelium of *A.aegypti*: they were mostly intracellular on the luminal side and intercellular near the haemocoel side (Torii *et al.*, 1992).

Ultrastructural studies of the encapsulation of *Plasmodium cynomolgi* in refractory *A.gambiae* females (Paskewitz *et al.*, 1988) indicate that this parasite also takes an intracellular route through columnar digestive cells with microvilli, and is encapsulated as it reaches the basal lamina. Degenerate cells, bearing *P.cynomolgi* melanotic capsules and completely detached from the epithelium, were observed free in the lumen of the midgut (Paskewitz *et al.*, 1988). Intracellular invasion of columnar cells with microvilli and a similar encapsulation process have been observed during EM studies of *P.berghei* invasion in the same mosquito strain (R.Cantera and F.C.Kafatos, unpublished).

The proposed model is very different from the one involving special, vATPase-overexpressing 'Ross cells', as determined for the *P.gallinaceum*–*A.aegypti* model system (Shahabuddin and Pimenta, 1998). It is possible that *P.gallinaceum* uses a mechanism of invasion different from that of mammalian *Plasmodium* species. However, two EM studies of *P.gallinaceum* invasion of *A.aegypti* midgut (Torii *et al.*, 1992; Vernick *et al.*, 1999) have documented parasites within the cytoplasm of columnar cells bearing microvilli and lacking the morphological characteristics described for the 'Ross cells' (minimal endoplasmic reticulum and lack of secretory granules).

We used seven independent criteria to conclude that in *A.stephensi* the cells overexpressing vATPase are not the cells invaded by ookinetes: (i) the invaded cells express the same levels of vATPase as uninvaded cells (Figure 2G); (ii) the invaded cells express high levels of NOS, while the cells with high vATPase levels do not express NOS (see inset in Figure 1C); (iii) the invaded cells measure 15–18 µm in diameter, while the vATPase-positive cells have a triangular shape with a maximum length of 8 µm (Figures 2C and H, and 3C and D); (iv) the main body of the infected cells is in contact with the midgut lumen, while the cells with high vATPase levels are flat and their cell body is in contact with the basal lamina and does not reach the lumen; (v) the protruding cells appear only in infected samples, indicating that their morphology is not that of a normal pre-existing cell (Figure 2A); (vi) parasites do not co-localize with cells expressing high levels of vATPase (Figure 2E); and (vii) the 'Ross cells' lack microvilli, while the invaded cells do have microvilli in their surface, albeit at a reduced density as compared with healthy cells (Figure 3C and D).

It is intriguing that the reported *A.aegypti* 'Ross cells' have two characteristics that are missing from the normal columnar epithelial cells, but that are observed in damaged cells: they protrude towards the lumen and are depleted of (or lack) microvilli. Is it possible that the observed overexpression of vATPase and the morphological characteristics of 'Ross cells' in *A.aegypti* are induced by injury to columnar epithelial cells? The observations leading to the description of the 'Ross cells' were made in an *in vitro* invasion system, in which the midguts were dissected, cut in half lengthwise, freed of blood contents, centrifuged with cultured ookinetes and incubated for 30 min *in vitro* before fixation. In our experimental system, even a fast dissection, followed immediately by

tissue fixation, induces injury in the cells along the incisions, resulting in a positive TUNEL assay. The extensive manipulation of the midguts during the *in vitro* invasion assay could have resulted in extensive cell damage and osmotic effects, complicating the interpretation of the results. A detailed morphological comparison of invaded and healthy uninvaded vATPase-overexpressing cells, *in vivo* as well as *in vitro*, at high magnification and utilizing several additional markers (actin, NOS and nuclear staining) would be required to compare the *P.gallinaceum*–*A.aegypti* system with that reported here for the *P.berghei*–*A.stephensi* system.

The proposed theory obviously has important implications for the design of potential transmission-blocking strategies. Further studies of the epithelial responses of different mosquito species infected with different parasites would be required to determine directly how universal the time bomb theory of invasion is. At present, there is no compelling evidence against the proposition that this invasion mechanism has been conserved across species.

Future perspectives

The time bomb theory also raises several questions. Is the parasite actively limiting the ability of the invaded cell to mount a defence reaction? If AsNOS could reach higher levels, be induced more quickly or for a longer period, would parasite invasion be blocked? Are lateral movements of ookinetes random events, or is the parasite capable of sensing that the NO levels are getting too high before reaching the basal lamina and using this strategy as a way of ‘buying time’? What are the effects of the proteins secreted by the parasite in the cytoplasm of the midgut cells?

Over the last few years, the number of characterized genes found to be involved in mosquito immune responses has increased rapidly. A further increase can be expected from the ongoing expressed sequence tag (EST) and future genome sequencing projects. One of the major present challenges is to develop new strategies to bridge the gap between sequence information and gene function. In this context, understanding the cell and molecular biology of parasite invasion becomes essential. We present this initial version of the time bomb theory of ookinete invasion as a reference point, to be challenged and modified as our understanding of the complex insect–vector interactions that determine vector competence evolves.

Materials and methods

Mosquitoes

Anopheles gambiae strain G3 was reared at 27°C, at 75% humidity on a 12 h light–dark cycle. Larvae were grown in distilled water with 0.01% table salt, and fed on cat food. Adults were fed *ad libidum* on 10% sucrose and females were blood-fed on anaesthetized mice. Larvae were challenged with *Escherichia coli* 1160 and *Micrococcus luteus* A270 as described (Richman, 1996).

In vitro ookinete culture

Ookinetes were obtained from infected mouse blood cultured *in vitro*, following the procedures previously described (Winger *et al.*, 1988). Briefly, Balb-C mice were treated with phenylhydrazine 3 days prior to infection with *P.berghei* and bled by cardiac puncture 3 or 4 days post-infection, when exflagellation was high. White blood cells were removed by passing the blood through a Plasmadipur® filter (Euro-Diagnostica, Arnhem, The Netherlands). The blood was then diluted 1:10 with

ookinete medium consisting of RPMI 1640 supplemented with L-glutamine and 0.025 M HEPES buffer (Gibco-BRL, Grand Island, NY), containing 10% heat-inactivated fetal calf serum (Gibco), 0.024 M NaHCO₃ and antibiotics (5 µg/ml penicillin, 5 µg/ml streptomycin and 1 µg/ml neomycin), adjusted to pH 8.4. After dilution, the blood was incubated at 19°C for 22 h.

Expression of a recombinant PbSub2 protein fragment

A *P.berghei* subtilisin (PbSub2) cDNA fragment was cloned by using degenerate primers designed based on the sequence alignment of other known members of the subtilisin family. The PCR product was used to screen a *P.berghei* ookinete cDNA library, and a partial cDNA clone was isolated and sequenced (J.Thompson, unpublished; DDBJ/EMBL/GenBank accession No. AAD33947). A 1.66 kb fragment encoding amino acids 686–1240 of PbSub2 was amplified from the *P.berghei* cDNA using primers AAGAGTATTTAATGCTTGGC and GTATTA-TCTCCTCCTTTTCTCG, and cloned sequentially into the pCR2.1 TOPO TA vector (Invitrogen) and the His tag protein expression vector pQE-30 (Qiagen). Recombinant protein was expressed in JM109 cells and purified under denaturing conditions using the manufacturer's recommended procedures.

Generation of polyclonal rat antisera

The *A.gambiae* vATPase F subunit cDNA had been cloned and sequenced previously (DDBJ/EMBL/GenBank accession No. Z69979). PCR primers were designed to amplify the coding region, which was cloned into a vector to generate a glutathione S-transferase fusion (M.Muskavitch, personal communication). Pure recombinant protein for antibody production was kindly provided by Dr Marc Muskavitch. The initial immunizations were performed by injecting two rats with a mixture containing 100 µg of ATPase protein in 100 µl of physiological saline and 100 µl of RIBI adjuvant (RIBI ImmunoChem Research, Inc.). Boosts consisted of 50 µg of the same protein mixture in RIBI adjuvant and were injected four times, beginning 3 weeks after injection and at 3 week intervals thereafter. The immune response of the rats was followed by testing several serum dilutions in western blots. The final polyclonal serum was specific, in that it detected a single band of the expected size (Figure 2H), not present when pre-immune sera were used.

Rat polyclonal anti-PbSub2 antisera were generated following the same procedures described above. The final sera recognized recombinant PbSub2, and specifically detected two major processed PbSub2 fragments of 64 and 58 kDa and a minor species of 130 kDa, corresponding to the PbSub2 precursor protein, on western blots of cultured *P.berghei* (ANKA) schizonts not detected by the pre-immune serum.

Midgut immunostainings

Midguts from mosquitoes fed on healthy or malaria-infected mice were dissected in phosphate-buffered saline (PBS; 130 mM NaCl, 7 mM Na₂HPO₄, 3 mM NaH₂PO₄·H₂O pH 7.2). The tissue was fixed for 30 s in 4% paraformaldehyde, 100 mM PIPES buffer pH 7.4, 2 mM MgSO₄ and 1 mM EGTA, opened longitudinally, and the luminal contents were removed in PBS and fixed for another 30 min at room temperature. The tissue was then blocked in 1% bovine serum albumin (BSA), 0.1% SDS in PBS pH 7.5 (PBT) for 2 h at room temperature. In between each of these steps, the tissue was washed twice for 5 min in PBS pH 7.5. For the peroxidase experiment, the midguts were incubated for 20 min at room temperature, either in PBS alone (–PV) or in PBS containing 1 mM sodium orthovanadate and 20 µM H₂O₂ (+PV) before fixation.

The Universal rabbit anti-NOS (Oncogene, Cambridge, MA), the rat vATPase and PbSub2 antibodies were diluted 1:300, and the mouse anti-Pbs21 antibody 1:600 in overnight incubations in PBT at 4°C. Secondary Cy5-, Cy3- (Amersham, Arlington Heights, IL) and Alexa488- (Molecular Probes, Eugene, OR) conjugated secondary antibodies were used at 1:1000 dilution in PBT for 3–4 h incubation at room temperature. After each antibody, the tissue was washed three times for 15 min with PBT. Actin was stained by using 6.6 µM Alexa488-conjugated phalloidin (Molecular Probes, Eugene, OR) in methanol diluted 1:40 in PBS with 1% BSA and incubated for 20 min at room temperature. The nuclei were counterstained with a 4',6-diamidino-2-phenylindole (DAPI) solution (1:15 000 dilution of stock solution, Boehringer Mannheim) for 2 min. Immunostaining was analysed by fluorescence microscopy, and nuclear DAPI staining by UV light illumination. The final images were obtained using either regular fluorescence or confocal microscopy with a Fluoview System (Olympus). Haemozoin pigment was visualized by detecting signal emitted into the red channel detector, when exciting the sample with the Ar laser (488 nm) in the absence of barrier filters.

TUNEL assay

DNA fragmentation was detected using the Fluorescein-FragEL Kit (Oncogene Research Products, Cambridge, MA), by modifying the suggested protocol for cell suspensions. Midguts were dissected, processed and fixed as described above. They were washed with TBS (20 mM Tris-HCl pH 8.0, 140 mM NaCl), followed by 5 min incubations in a TBS-methanol step gradient from 0, 25, 50, 75 to 100% methanol and back to TBS buffer following the same steps. The tissue was then incubated for 2 min at room temperature with 10 µg/ml proteinase K in TBS and the reaction stopped by adding 10 vols of 2 µg/ml glycine. This was followed by a TBE wash and a 10 min fixation with 4% paraformaldehyde in TBS. The sample was equilibrated for 20 min with terminal transferase buffer and then labelled for 1 h at 37°C. The tissue was counterstained with DAPI and mounted.

Acknowledgements

We would like to thank Dr Marc Muskavitch for providing recombinant *A. gambiae* vATPase protein, Dr Mohammed Shahabuddin for making the *A. aegypti* vATPase serum available, and Dr Benjamin Wizel for suggesting the TUNEL assay. This work was funded by a Faculty Research Grant and a College Research Council Grant from Colorado State University, and by a Research Grant from the National Institutes of Health No. AI5573-01A1. Early stages of the work were funded by the John D. and Catherine T. MacArthur Foundation.

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*Received July 24, 2000; revised September 26, 2000;
accepted September 27, 2000*

Note added in proof

Zieler and Dvorak [*Proc. Natl Acad. Sci. USA* (2000), **97**, 11516–11521] have now reported an invasion study of *A.aegypti* midgut epithelial cells by *P.gallinaceum*, using video microscopy in an *in vitro* system. Their results indicate that ookinete invasion induces cell death, probably by an apoptotic mechanism. Their results are complementary and in agreement with those presented in this manuscript, thus supporting the idea that the mechanisms of ookinete invasion have been conserved across species.