The Journal of Experimental Biology 204, 2773–2780 (2001)
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JEB3509

MALARIA-INDUCED APOPTOSIS IN MOSQUITO OVARIES: A MECHANISM TO CONTROL VECTOR EGG PRODUCTION

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Accepted 29 May 2001

Summary

Many insects are able to adjust their egg production according to physiological conditions such as nutrient supply and mating success. One way in which this is achieved is by resorption of some, or all, of the ovarian follicles at some stage during oogenesis. We have shown that the mosquito *Anopheles stephensi* responds in this manner when ookinetes of the malaria parasite *Plasmodium yoelii nigeriensis* first begin to invade the midgut. Little is known about the initiation and regulation of follicle resorption in any insect. Here, we demonstrate that there is a significant positive correlation between follicle resorption and the presence of follicular epithelial cells that are undergoing apoptosis. The parasite causes significantly more follicles to contain apoptotic cells from 16 h post-infection onwards. Injection of a caspase inhibitor immediately after feeding on an infective blood meal prevents parasite-induced resorption of follicles and thus demonstrates that apoptosis precedes resorption. Ultrastructural studies show that patches of follicular epithelial cells contain condensed nuclear chromatin, a characteristic of apoptosis, and that no patency develops in these cells. Our work suggests that apoptosis plays a role in malaria-initiated inhibition of mosquito oogenesis and that caspase is central to this process. Follicle resorption is one of the main factors contributing to malaria-induced fecundity reduction in mosquitoes.

Key words: mosquito, *Anopheles stephensi*, oogenesis, follicle resorption, apoptosis, caspase, *Plasmodium yoelii nigeriensis*, malaria, follicular epithelium, nurse cell.

Introduction

Fecundity reduction is a life-history trait commonly associated with parasitized molluscs and arthropods (Hurd, 1990). It is thought to occur as a result of a trade-off in host resource management between demands made by the parasite and those required for egg production (Obrebski, 1975; Read, 1990). Complete castration rarely occurs and, in insects in particular, some temporary or permanent curtailment of egg production is most common (Hurd, 1993). Although there are numerous reports of the effect of parasites on insect reproductive success, our understanding of the mechanisms that initiate and control fecundity reduction is poor. We are currently examining the effects of malaria on various aspects of oogenesis in the major vectors of human malaria *Anopheles stephensi* and *Anopheles gambiae*.

Malaria is regarded as the most serious of the parasitic diseases, causing 300–500 million clinical cases and 1.5–2.7 million deaths annually (www.who.org). It is caused by protozoans of the genus *Plasmodium* and is transmitted by anopheline mosquitoes during blood feeding. Within 24 h of ingestion, *Plasmodium* spp. gametes are fertilised in the blood bolus within the mosquito midgut, and motile ookinetes, developed from zygotes, begin to traverse the midgut wall. They transform into oocysts below the basal lamina of the midgut epithelium (Beier, 1998). These oocysts produce sporozoites that invade the salivary gland, ready to infect another vertebrate host during the next blood meal. These developmental stages of the rodent malaria *Plasmodium yoelii nigeriensis* cause a significant reduction in the number of eggs produced by *Anopheles stephensi* and *Anopheles gambiae* following successive blood meals (Hogg and Hurd, 1995a; Hogg and Hurd, 1995b; Ahmed et al., 1999). Thus, the impact of infection upon mosquito reproductive fitness is cumulative.

Mosquito meroistic ovaries contain a series of approximately 50 ovarioles that radiate from a calyx. Each ovariole consists of a germarium and two follicles or egg chambers. The latter are composed of one oocyte and seven nurse cells and are enclosed in a follicular epithelium (Sokolova, 1994). Egg production is cyclical and synchronous. A blood meal initiates the maturation of the proximate follicles, resulting in oviposition of an egg batch at the end of a gonotrophic cycle. During this process, the follicular epithelial cells become patent and separate to allow passage of

yolk protein precursors, including vitellogenin, into the oolemma, where it is internalised by receptor-mediated endocytosis and deposited in a crystalline form, vitellin (Snigirevskaya et al., 1997; Raikhel and Snigirevskaya, 1998). In *Anopheles stephensi*, yolk protein accumulates in the terminal follicles from trace levels at 4 h post-bloodmeal to a maximum in 2 days (Hogg et al., 1997). Nurse cells synthesise RNA and other macromolecules that are transported to the oocyte cytoplasm *via* intercellular canals. By 48–50 h, oocyte maturation is completed; epithelial cells have secreted a chorion (egg shell) and the nurse cells degenerate. Events surrounding the degeneration of the nurse cells and follicular epithelium are not well-documented.

When a female mosquito feeds on host blood containing malaria gametocytes, the gonotrophic cycle begins normally. However, soon after vitellin begins to accumulate in the terminal follicles, a significant proportion of them undergo resorption (Carwardine and Hurd, 1997). This process reduces the size of the final egg batch produced and is the major contributory factor to *Plasmodium*-induced fecundity reduction. To understand the parasite-induced signals that initiate and control fecundity reduction, we are studying the process of follicle resorption in infected mosquitoes.

The signalling mechanisms and cellular processes involved in uninfected-insect follicle resorption are poorly understood. Nevertheless, we thought it likely that malfunctioning cells of the follicular epithelium might be involved in initiating the process because they play a key role in the passage of vitellogenin into the oocyte in mosquitoes. We have designed experiments to investigate this hypothesis. Alternatively, follicular epithelial cells could play an active part in resorption by enzyme degradation and phagocytosis of accumulating yolk, as seen in locust resorbing follicles (Lüsis, 1963). Here, we report that malaria infection induces apoptosis in cells of the follicular epithelium and that this programmed cell death results in follicle resorption.

Materials and methods

Mosquito maintenance and infection

Larvae of *Anopheles stephensi* (Dubai) Liston were reared under standardised conditions to produce adult females of similar size (Jahan and Hurd, 1998), and mosquitoes emerging within the same 24 h period were kept in cages of 27 000 cm^3. Adults were maintained at 26±1 °C, 80 % relative humidity in a 12 h:12 h light:dark cycle and provided with 10 % glucose with antibiotics (2.8 ml l^{-1} of a stock solution of 10 000 i.u. penicillin and 10 mg ml^{-1} streptomycin in 0.9 % sodium chloride) and 0.05 % para-aminobenzoic acid to enhance parasite development (Peters and Ramkaran, 1980).

Male CD mice were infected with *Plasmodium yoelii nigeriensis* strain N67 following one serial passage of blood from mice infected from cryopreserved stocks (Hogg and Hurd, 1995b). Parasitaemia and gametocytaemia were monitored in Giemsa-stained, thin blood films and unstained, fresh, thick blood films, respectively, at 24–30 h post-infection.

Nulliparous female mosquitoes (6–8 days post-emergence) were allocated randomly to two groups and starved for 12–18 h before feeding on anaesthetized non-infected mice or infected mice (gametocytaemic and exflagellating) 24–30 h post-infection (Jahan and Hurd, 1998). The packed cell volumes of control and infected mice (litter siblings) were measured immediately prior to blood-feeding the mosquitoes. The control mouse was selected to have a packed cell volume within 2 % of that of the infected mouse to ensure that bloodmeal haemoglobin content was not reduced in infected mosquitoes (Taylor and Hurd, 2001). For each study, a group of engorged females that had fed on the infected mouse was separated and retained to check for prevalence and intensity of infection by monitoring oocysts developing on the midgut at day 7 post-bloodmeal. Prevalence of infection always exceeded 90 %, and oocyst burden was always over 50 per mosquito (data not shown). The wing length of each mosquito used in the study was measured as an assessment of mosquito size (Jahan and Hurd, 1997). No significant differences between control and infected groups in individual experiments were found (data not presented).

Follicle resorption and apoptosis in whole ovaries

Fully engorged female mosquitoes were selected at random and assigned to groups for examination at 16, 18, 20, 22, 24 and 36 h post-bloodmeal. All groups were maintained in the insectary conditions described above and chilled for 2–3 min at –20 °C prior to dissection. Ovaries were dissected in *Aedes* physiological saline (APS) (150 mmol l^{-1} NaCl, 4 mmol l^{-1} KCl, 1 mmol l^{-1} $CaCl_2$, 0.1 mmol l^{-1} $NaHCO_3$, 0.6 mmol l^{-1} $MgCl_2$ buffered with 25 mmol l^{-1} Hepes) and dipped in Neutral Red [Gurr, London; 0.5 % (w:v) solution in citrate-phosphate buffer: 0.1 mol l^{-1} citric acid/0.1 mol l^{-1} sodium citrate] at pH 6 for 1 min to assist the visualization of follicles (Clements and Boocock, 1984). Neutral Red concentrates in lysosomes and yields a deep red colour at a slightly acid pH (Wilson, 1990). Resorbing follicles exhibit an increased uptake of Neutral Red (Bell and Bohm, 1975; Clements and Boocock, 1984). Ovaries were further dissected into individual ovarioles and examined under a dissecting microscope at ×100 (Carwardine and Hurd, 1997). The number of resorbing follicles was recorded. Acridine Orange (50 µmol l^{-1} in APS) was then added to the ovaries, and they were immediately examined using fluorescence filters. The number of follicles containing epithelial cells with condensed nuclei was counted. Acridine Orange is one of several nucleic acid stains routinely used to reveal the condensed chromatin characteristic of apoptotic cells (e.g. McGahon et al., 1995; Longthorne and Williams, 1997).

Detection of DNA fragmentation in ovarian tissue

Apoptotic nuclei were further identified in paraffin-wax-embedded sections of ovaries by *in situ* terminal-deoxynucleotidyl-transferase-mediated dUTP-biotin nick end labelling (TUNEL) using the Oncogene Research Products TdT-FragEL DNA fragmentation detection kit. The procedure was carried out according to the manufacturer's instructions, and sections were counterstained with Methyl Green to aid the

morphological evaluation and characterization of normal and apoptotic cells. Mosquitoes were infected and treated as outlined above. At each time point, ovaries were dissected in APS then immediately dehydrated in a graded ethanol series prior to embedding in wax. The objective here was to confirm that cells of the follicular epithelium were undergoing apoptosis and to check whether nurse cells, which were not visible in whole-mounts, were also apoptotic. The TUNEL technique identifies the presence of DNA fragmentation, an early stage in the process of programmed cell death. In total, 50 sections were observed, and attention was focused on ovaries from infected mosquitoes during the early stages of oogenesis, although some sections from uninfected mosquitoes were examined. The sampling technique thus precluded statistical comparisons between time points or between infected and uninfected females.

Ultrastructural examination of ovaries from infected mosquitoes

Ovaries from uninfected and infected females (treated as above) were dissected in APS and fixed in 3% (v:v) glutaraldehyde in 0.1 mol l^{-1} sodium phosphate buffer, pH 7.4, and post-fixed in 1% (w:v) osmium tetroxide in the same buffer for 1 h before dehydration in a graded ethanol series and embedding in Spurr's resin. Ultrathin sections were stained with uranyl acetate and lead citrate and observed with a Joel JEM 100CX11 transmission electron microscope. Grids from each time point were examined at a series of magnifications from ×1900 to ×7200.

Inhibition of apoptosis

To establish conclusively that malaria-induced apoptosis caused follicle resorption, we attempted to inhibit the activation of apoptosis, using a caspase inhibitor [z-VAD.fmk, benzyloxycarbonyl-Val-Ala-Asp (Ome) fluoromethylketone; Garcia-Calvo et al., 1998; Rodriguez et al., 1996; Jacobson et al., 1996]. Immediately after blood feeding on a gametocytaemic mouse, fully engorged females were injected with 0.25 µl of z-VAD.fmk [0.5 mmol l^{-1} in a solution of dimethyl sulphoxide (DMSO) 1:100 (v:v) in APS], with DMSO [1:100 (v:v) APS] or with APS alone. Solutions were injected into the thorax just below the base of a wing using a finely drawn out, calibrated glass microcapillary tube. A further control group of uninjected, blood-fed mosquitoes was maintained under identical conditions. All mosquitoes emerged on the same day and were fed on the same infected mouse.

Mosquitoes were returned to the insectary and given access to sugar solution (as above) for 24 h. At this time, a comparison of the number of resorbing follicles in ovaries from mosquitoes subjected to each treatment was made.

Results

Ovaries from infected mosquitoes contained significantly more resorbing follicles and exhibited more signs of apoptosis

Both ovaries from 20 infected and 20 uninfected females were examined at each of six times post-bloodmeal (Fig. 1).

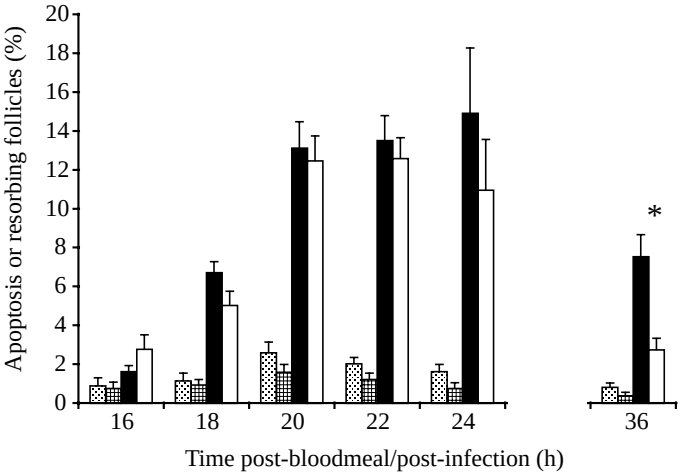

Fig. 1. The effect of malaria infection on the occurrence of apoptosis and resorption of follicles in ovaries from *Anopheles stephensi*. Mean percentage of follicles per ovary stained with Neutral Red and thus resorbing; uninfected mosquitoes (filled squares), infected mosquitoes (filled columns) and/or containing apoptotic nuclei stained with Acridine Orange; uninfected mosquitoes (open squares), infected mosquitoes (open columns). Sample size, 20 in each case. Values are means + S.E.M. A significant difference occurred between resorption and apoptosis in infected females at one time point (*P<0.05).

Resorbing follicles, detected by general appearance and intense staining with Neutral Red (Clements and Boocock, 1984), were first evident 16 h after the gonotrophic cycle had been initiated. In uninfected mosquitoes, maximum resorption was observed at 20 h post-bloodfeeding and never exceeded 4% of the total follicles. Infection induced a significant overall increase in resorption at all times examined, and the proportion of resorbing follicles continued to rise after 20 h. Fewer resorbing follicles could be detected by 36 h post-bloodmeal (Fig. 1).

Acridine Orange staining of the same ovaries demonstrated that a similar proportion of follicles contained cells that were

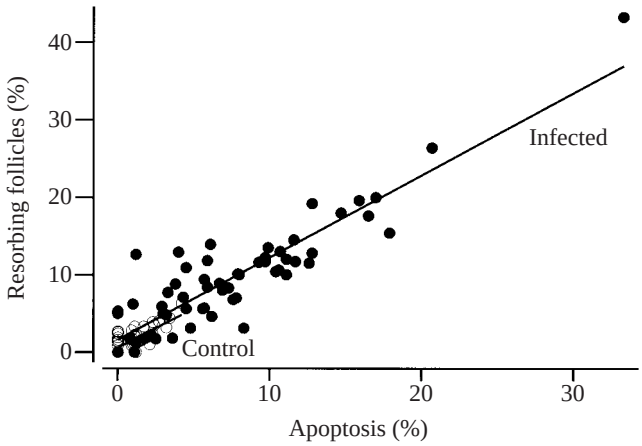

Fig. 2. The relationship between resorption and apoptosis for control and infected mosquitoes (open circles, control: 58 d.f.; r^2=0.569; P<0.001; filled circles, infected: 58 d.f.; r^2=0.807; P<0.001).

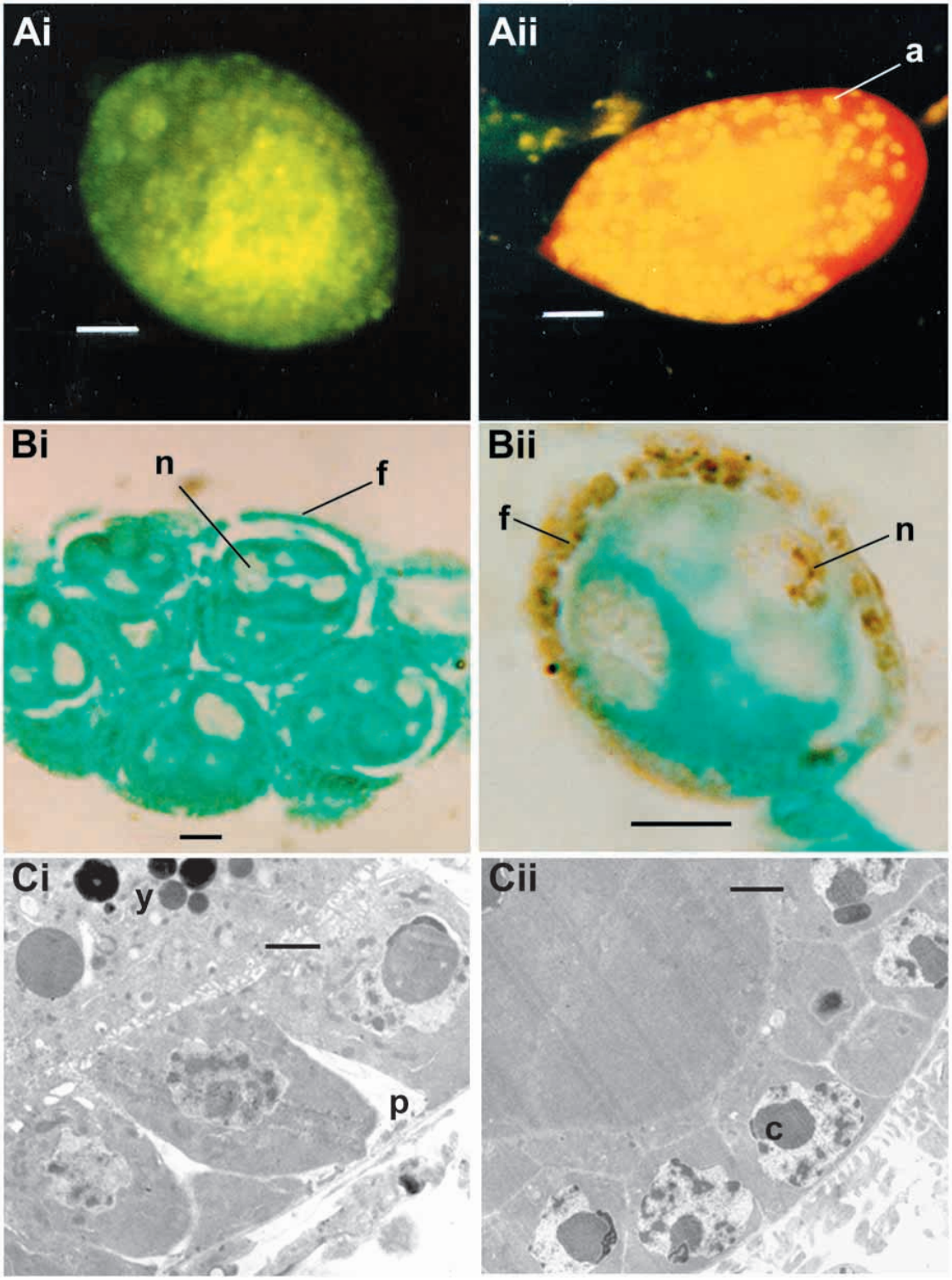

Fig. 3. Follicles from ovaries of malaria-infected *Anopheles stephensi* developing normally (left) or showing evidence of apoptosis (right). (A) Whole-mounts of individual follicles 24 h post-bloodfeeding, viewed under ultraviolet light after staining with Acridine Orange. The follicle in the left-hand frame (Ai) is exhibiting autofluorescence, and condensed apoptotic nuclei are visible in the follicle on the right, stained orange (a) (Aii). Scale bars, 20 μm. (B) Sections of follicles from ovaries 16 h post-bloodfeeding stained with Methyl Green and treated by TUNEL (see Materials and methods). DNA fragmentation (indicated by brown staining) can be observed in follicular epithelial (f) and nurse (n) cells in the right-hand frame (Bii). Scale bars, 10 μm. (C) Electron micrographs of a normal follicle 18 h post-bloodfeeding (Ci) and a resorbing follicle 22 h post-bloodfeeding (Cii). Patency (p) has developed in follicular epithelial cells of the normal follicle, and yolk spheres (y) have been deposited, whereas in the resorbing follicle no yolk is visible, epithelial cells are not patent and their nuclei contain condensed chromatin (c). Scale bars, 2 μm.

undergoing apoptosis to those that were resorbing (see Fig. 3A). A two-way analysis of variance (ANOVA), carried out on arcsine-transformed data, showed an overall increase in the number of resorbing follicles in infected *versus* uninfected mosquitoes ($F_{1,108}$=222.25; P<0.001) and an overall increase in apoptosis in follicles from infected compared with control mosquitoes ($F_{1,108}$=199.10; P<0.001).

A Tukey's unplanned comparison between pairs of means demonstrated that a significant difference occurred between resorption and apoptosis only in infected females at 36 h postfeeding (P<0.05). The reduction in the occurrence of apoptosis at 36 h (Fig. 1) was to be expected if apoptosis, which is a short-lived event, occurred prior to follicle resorption. That there is a relationship between resorption and apoptosis is shown by a three-way ANOVA on percentage resorption using a general linear model with percentage apoptosis as covariate, which indicates a significant relationship between the two ($F_{1,96}$=32.50; P<0.001) irrespective of whether the mosquitoes are infected or uninfected, except at 36 h. However, as stated above, by this time most of the apoptosis has already occurred (Fig. 2).

Apoptosis occurred in the cells of the follicular epithelium

Patterns of nuclei stained with Acridine Orange suggested that apoptosis was occurring in follicular epithelial cells (Fig. 3A). We confirmed this by examination of ultrathin sections of ovaries from infected females. Condensed nuclear chromatin, a characteristic of apoptosis, was seen in adjacent cells of the follicular epithelium of many terminal follicles of ovarioles dissected at the same time points post-bloodmeal as were used for Acridine Orange staining. No development of patency had occurred in these apoptotic cells by 22 h post-bloodmeal, and normal yolk spheres were not evident in oocytes surrounded by apoptotic cells (Fig. 3C). It appears that the occurrence of a patch of cells undergoing apoptosis may prevent vitellogenin gaining access to the adjacent oolemma.

DNA fragmentation was detected very early in the infection process

The initial stage of apoptosis is characterised by DNA fragmentation, an early event, that is sometimes detectable before the production of gross apoptotic morphology. Using TUNEL, we were able to detect the onset of apoptosis in patches of follicular epithelial cells in ovaries from infected females 16 and 18 h post-bloodmeal. We also observed that some labelled follicles contained nurse cells with apoptotic nuclei at these times (Fig. 3B). Fragmentation of DNA was not detected in the follicles examined at 22, 24 and 36 h post-bloodmeal. This aspect of the study was designed to confirm that the Acridine Orange staining we had previously observed demonstrated that apoptosis was taking place in the follicular epithelium. It also enabled us to examine more tissue than was feasible using electron microscopy because each section contained several follicles. No quantitative comparison between uninfected and infected ovaries was undertaken using TUNEL because of our choice of sampling method, and we did not observe any staining in the few sections from uninfected mosquitoes that were examined, probably because of the low frequency of apoptotic follicles in uninfected mosquitoes. It is possible that DNA fragmentation is completed by 22 h or that, because of the low frequency of follicles with cells in this early stage, they were not included in our sample.

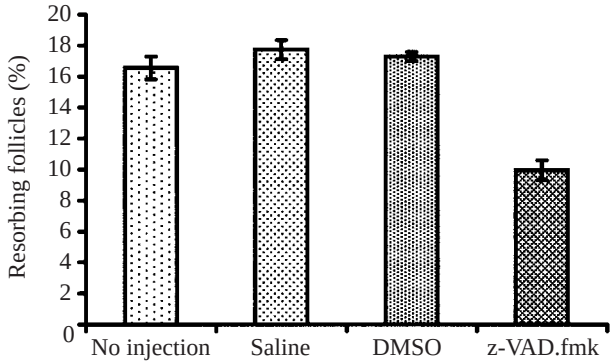

Fig. 4. The effect of inhibiting apoptosis on follicle resorption in malaria-infected *Anopheles stephensi*. Immediately after feeding on a mouse containing *Plasmodium yoelii nigeriensis* gametocytes, mosquitoes were injected with saline (N=11), 1 % DMSO in saline (N=25) or 0.5 mmol l^{-1} z-VAD.fmk/1 %DMSO (N=20) or left uninjected (N=20). The mean number of resorbing follicles per ovary pair is shown. Values are means ± S.E.M. A Kruskal–Wallis test demonstrated overall significance (P<0.001) followed by Mann–Whitney U-tests where all comparisons against z-VAD.fmk were significant (P<0.001). There was no significant difference between non-injected, saline-injected and DMSO-injected females.

Caspase inhibition prevents follicle resorption

Administration of the caspase-inhibitor z-VAD.fmk to infected mosquitoes significantly reduced the proportion of follicles undergoing resorption at 24 h post-bloodmeal from 16 %, in untreated females, to 10 %. Ovaries from untreated females had a similar number of resorbing follicles to those in previous experiments, and injection of the inhibitor-solvent or saline alone did not reduce this proportion (Fig. 4).

Discussion

In common with many organisms, insects are able to adjust their investment in reproduction in accordance with environmental and physiological conditions (Price, 1984). One of the major methods of achieving this adjustment is *via* atresia of follicles at some time during their development. This has been observed in many uninfected insects, but the mechanisms that initiate and control it are poorly understood (Bell and Bohm, 1975). In *Drosophila melanogaster* and other insects, including mosquitoes, it has been attributed to nutritional stress or lack of mating success (Clements and Boocock, 1984; Soller et al., 1999). Follicle resorption has also been associated with metacestode infections of the beetle *Tenebrio molitor* (Hurd and Arme, 1987).

In our studies of malaria-infected mosquitoes, females are

mated before they are infected, and thus an indirect effect *via* changes in the transfer of, or response to, sex peptides will not be taking place. Nor do we think that nutrient stress resulting from infection is the prime cause. Using our experimental regime, no decrease in haemoglobin intake occurs in mosquitoes feeding on infectious mice (Jahan et al., 1999; Taylor and Hurd, 2001), and the protein content of the blood meal is digested normally (Jahan et al., 1999). In addition, follicle resorption begins at a time when ookinetes are invading the midgut, and nutrient competition between parasite and host is probably not occurring so early in infection. As part of our investigations to determine whether there is a mechanism controlling oogenesis that is unique to infection, or whether the malaria parasite reduces egg production *via* a route associated with other physiological or environmental triggers, we need to understand the process of follicle resorption in mosquitoes.

We have now established that the programmed cell death of large patches of follicular epithelial cells is an early event in malaria-induced resorption of developing follicles and that apoptosis precedes resorption. Although we observed DNA fragmentation occurring in some of the nurse cells in resorbing follicles, the relative role of these cells is unclear. Our observation that follicular atresia is also associated with apoptosis in a second malaria-infected mosquito species, *Anopheles gambiae* (data not shown), suggests that this may be a universal response to malaria infection.

The occurrence of apoptosis in mosquito tissue has only recently been recorded. It was observed in studies of the midgut cells during metamorphosis (Nishiura and Smouse, 2000) and malaria ookinete invasion (Han et al., 2000), but no previous report of a role for programmed cell death in any aspect of mosquito oogenesis has been made. This is in contrast to *D. melanogaster*, in which studies of oogenesis are much more advanced. Apoptosis has been reported to occur in *D. melanogaster* nurse cells following cytoplasmic dumping, and the expression of *D. melanogaster* inhibitors of apoptosis, DIAP1 and DIAP2, in nurse cells ceases during stages 7 and 8 (Foley and Cooley, 1998). Soller et al. (Soller et al., 1999) suggested that regulatory switches for oogenesis reside in the ovary and operate at stage 9–10, which may be an apoptosis-sensitive window. Apoptosis of nurse cell nuclei occurred early in this process, but no Acridine Orange staining of the follicle cell nuclei occurred nor did they label with TUNEL (Soller et al., 1999). However, clones of defective follicle cells cause degeneration of the whole follicle (M. Bownes, personal communication). Thus, different processes may occur in *D. melanogaster* nurse and follicular epithelium cells. Furthermore, apoptosis in *D. melanogaster* follicle cells is induced by ectopic expression of *reaper* and *hid* transgenes and can be suppressed by co-expression of the baculovirus p35 protein. In these experiments, substantial death of follicle cells resulted in germline death (Cho and Nagoshi, 1999).

Our observation of malaria-induced resorption of anopheline mosquito follicles has demonstrated that nuclear disintegration and chromatin condensation occur in large groups of follicular epithelial cells as well as in some nurse cells. The process of follicle resorption and the role of apoptosis in normal oogenesis may, therefore, differ between *D. melanogaster* and mosquitoes. In particular, there is no single stage during mosquito oogenesis at which follicle resorption occurs (Clements and Boocock, 1984), and follicular epithelial cells may play a more prominent role than nurse cells.

The caspases (Alnemri et al., 1996) are the biochemical core of the apoptosis mechanism. They are site-specific proteases acting in a cascade to bring about the subcellular changes characteristic of apoptosis (e.g. DNA fragmentation, chromosomal condensation and stimulation of phagocytosis; Hengartner, 2000). At least five caspases have been identified so far in *D. melanogaster*. The caspase-encoding genes *dcp*-1 and *decay* are involved in *D. melanogaster* nurse cell apoptosis, which occurs during normal oogenesis (Dorstyn et al., 2000; McCall and Steller, 1998). Our work demonstrates that caspases are involved in mosquito follicle cell apoptosis and that inhibition of caspase activity stops resorption. This supports our premise that programmed cell death of follicular epithelial cells initiates resorption. However, at this stage, we do not know whether the *D. melanogaster decay*-like gene is involved.

Ovaries in *Plasmodium*-infected mosquitoes are responding to the presence of resorption-inducing signals that may, or may not, be specific to this infection. The nature of these signals is, however, currently unknown. There is evidence from the literature that the hormonal milieu influences insect follicle maintenance/degeneration involving apoptosis (Capella and Hartfelder, 1998). The balance between the insect steroid hormone ecdysone (secreted by mosquito ovaries) and juvenile hormone may be important, as in *D. melanogaster*. Soller and colleagues (Soller et al., 1999) proposed a model for oocyte maturation in *D. melanogaster* that identifies the balance between two hormones, 20-hydroxyecdysone and juvenile hormone, as playing a key role. Sex-peptide, transferred to females during mating, inhibits the apoptosis-inducing effect of 20-hydroxyecdysone during early vitellogenesis and prevents resorption at this time. Treatment of *D. melanogaster* with 20-hydroxyecdysone resulted in the resorption of some egg chambers. This particular hormonal balance may not, however, be important in mosquitoes. In uninfected mosquitoes, ecdysteroid titres peak midway through the gonotrophic cycle, when juvenile hormone levels are low (for a review, see Clements, 1992). Thus, high ecdysone/low juvenile hormone titres in the haemolymph may not initiate apoptosis/resorption. Instead, changes in local hormone synthesis by individual follicles may be involved in mosquitoes. At present, we do not know whether infection alters hormone titres, but there is some evidence that the endocrine system is a possible signalling mechanism between *Plasmodium* and mosquito tissue (Ysikevich and Zvantsov, 1999), and this may have a role in regulating programmed cell death.

Throughout the initial and subsequent gonotrophic cycles post-infection, yolk protein accumulates in the haemolymph of

infected mosquitoes as the rate of uptake by the ovaries declines as a result of follicle resorption (Hogg et al., 1997; Ahmed et al., 2001). In addition, infection results in a significant reduction in the abundance of vitellogenin mRNA that is likely to result in a reduction in vitellogenin synthesis (Ahmed et al., 2001). In infected females, resources that under normal circumstances would have been devoted to reproduction are being diverted away from egg production. This is a common parasite strategy that may exploit a trade-off between egg production and longevity by manipulation of host resource management (Koella, 1999). An understanding of the underlying physiological mechanisms and the fitness advantages to parasite and vector will help to clarify the adaptive nature and significance of host fecundity reduction (Read, 1990; Hurd and Webb, 1997; Hurd, 2001).

We have now shown that apoptosis is an important feature of malaria/mosquito interactions because it seems to precipitate the process that results in fecundity reduction. This exploitation of the physiological mechanisms of programmed cell death appears to result from a signal generated by the parasite, directly or indirectly, as it invades the midgut and stimulates an immune response (Richman et al., 1997; Luckart et al., 1998). This signal may be long-lasting, thus affecting subsequent cycles of egg production, or new signals, of the same or different origin, may occur at later stages of infection. Further investigation of this phenomenon should produce a greater understanding of the diverse factors controlling malaria transmission *via* mosquitoes and shed light on the normal processes involved in the regulation of mosquito egg production.

We gratefully acknowledge funding from the Egyptian Government Scholarship Award Programme (A.M.A.) and the Medical Research Council (G.T.W.).

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