Malaria parasite exit from the host erythrocyte: A two-step process requiring extraerythrocytic proteolysis

Brandy L. Salmon, Anna Oksman, and Daniel E. Goldberg*

Howard Hughes Medical Institute, Departments of Molecular Medicine and Microbiology, Washington University School of Medicine, St. Louis, MO 63110

Edited by Anthony Cerami, The Kenneth S. Warren Laboratories, Tarrytown, NY, and approved October 30, 2000 (received for review August 29, 2000)

Intraerythrocytic malaria parasites replicate by the process of schizogeny, during which time they copy their genetic material and package it into infective merozoites. These merozoites must then exit the host cell to invade new erythrocytes. To better characterize the events of merozoite escape, erythrocytes containing *Plasmodium falciparum* schizonts were cultured in the presence of the cysteine protease inhibitor, L-transepoxy-succinyl-leucylamido-(4-guanidino)butane (E64). This treatment resulted in the accumulation of extraerythrocytic merozoites locked within a thin, transparent membrane. Immunomicroscopy demonstrated that the single membrane surrounding the merozoites is not erythrocytic but rather is derived from the parasitophorous vacuolar membrane (PVM). Importantly, structures identical in appearance can be detected in untreated cultures at low frequency. Further studies revealed that (*i*) merozoites from the PVM-enclosed merozoite structures (PEMS) are invasive, viable, and capable of normal development; (*ii*) PEMS can be purified easily and efficiently; and (*iii*) when PEMS are added to uninfected red blood cells, released merozoites can establish a synchronous wave of infection. These observations suggest that L-transepoxy-succinyl-leucylamido-(4-guanidino)butane (E64) causes an accumulation of an intermediate normally present during the process of rupture. We propose a model for the process of rupture: merozoites enclosed within the PVM first exit from the host erythrocyte and then rapidly escape from the PVM by a proteolysis-dependent mechanism.

Malaria afflicts an estimated 300 million to 500 million people and is responsible for the death of nearly 2 million children each year (1). Malaria is caused by several species of obligate intracellular protozoan parasites, the most deadly of which is *Plasmodium falciparum*. The malaria parasite completes its asexual life cycle in the mature human erythrocyte within a vacuole, delimited by the parasitophorous vacuole membrane (PVM). While in the red cell, the parasite undergoes three distinct stages of development: the ring, the trophozoite, and the schizont stage. During schizony, the parasite divides within the vacuole to form between 16 and 32 merozoites. Approximately 48 h after infection, the merozoites rupture from within the red cell to invade new cells and continue the cycle.

In a 1986 study by Lyon and Haynes (2), purified *P. falciparum* schizonts that were cultured in the presence of small concentrations of leupeptin and chymostatin failed to properly rupture and, instead, formed "protease inhibitor clusters of merozoites." A number of proteases are suspected to be involved in parasite exit, based on circumstantial evidence. The stage-specific expression of a cysteine protease (35,000–40,000 M_r) and a serine protease (75,000 M_r) found in mature schizonts and merozoites, respectively, implicates them in the process of rupture or invasion (3). The *P. falciparum* serine repeat antigen, known as SERA, P126, SERA-1, and SERP, is one of several homologues found on chromosome 2 (4–8). Because SERA and its homologues contain an approximately 30-kDa protease domain with similarity to papain-like cysteine proteases and some are known to be expressed in late schizonts, they have been implicated in events of rupture or invasion as well. Interestingly, P126 (SERA) has been shown to be a major component of the parasitophorous vacuole in late erythrocytic stages and is proteolytically processed upon release of merozoites into culture medium—processing that is altered with the addition of the protease inhibitor leupeptin (4, 9, 10). The aspartic protease plasmepsin II has also been shown to be secreted to the parasite periphery in late schizonts and is active in degrading spectrin (11). The precise roles of these various proteases in erythrocyte rupture and parasite escape have not been established.

Aside from the fact that proteases are likely to be involved in rupture, relatively little is known about the event. In a recent study by Winograd *et al.* (12), video microscopy was performed on late schizonts undergoing rupture. The study found that the merozoites were released through an opening in the host erythrocyte, and the release of merozoites was not mediated by an explosive event. In the current study, effects of the protease inhibitor E64 are assessed, and a model for rupture is proposed.

Materials and Methods

Strains, Growth Conditions, and Media. Parasite clones used in this study were kindly provided by W. Trager, The Rockefeller University, New York (HB3, Honduras); T. Wellems, National Institutes of Health, Bethesda, MD (Dd2, The Netherlands); and P. Rathod, Catholic University, Washington, DC (3D7 and W2, Indochina). Parasites were cultured by using the method of Trager and Jensen (13) and were maintained under 3% O_2/3% CO_2/94% N_2. RPMI medium 1640 was supplemented with 25 mM Hepes (Sigma)/30 mg/liter hypoxanthine (Sigma)/0.225% $NaHCO_3$ (Sigma)/0.5% Albumax I (Life Technologies, Grand Island, NY). Parasites were synchronized by using sorbitol treatment (14).

Protease Inhibitor Treatment. All experiments shown were performed with the HB3 strain of *P. falciparum*; however, inhibitor assays were also performed by using 3D7, W2, and Dd2 to rule out strain-specific effects. Parasites were grown in O+ human red blood cells at 2% hematocrit and were synchronized at least twice by sorbitol treatments. Parasitized cells were then grown in normal culture medium until middle-stage schizonts were obtained, each containing between five and seven nuclei. Synchronous cultures were treated with 10 μM of freshly prepared E64 (Sigma) for approximately 8 h and then viewed by indirect immunofluorescence assay, Giemsa stain, or electron microscopy or subjected to additional purification procedures. Syn-

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: E64, L-transepoxy-succinyl-leucylamido-(4-guanidino)butane; PVM, parasitophorous vacuolar membrane; PEMS, PVM-enclosed merozoite structures.

*To whom reprint requests should be addressed. E-mail: goldberg@borcim.wustl.edu.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Article published online before print: *Proc. Natl. Acad. Sci. USA*, 10.1073/pnas.011413198. Article and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.011413198

chronous cultures of untreated schizonts served as controls and were either harvested 6 h after initiation of experiment (i.e., addition of E64 to corresponding treated cultures) to harvest late schizonts (8–16 nuclei) or, in some experiments, allowed to progress to late schizonts (16–32 nuclei) and early rings.

Indirect Immunofluorescence Assay. Control cultures and cultures treated with E64 for approximately 8 h were collected by centrifugation and washed three times in PBS. Cells were smeared on glass coverslips, allowed to air-dry, and fixed in acetone for 5 min at room temperature, in methanol for 10 s at 4°C, or in 1% formaldehyde for 15 min at room temperature. Samples spotted without smearing gave similar results. All three fixation methods yielded similar results. Samples shown herein were fixed in 1% formaldehyde. After fixation, samples were washed three times in PBS and then blocked in 10% FBS (Life Technologies) in PBS for 30 min at 37°C. Samples were then incubated in primary antibodies diluted in blocking buffer for 1 h at 37°C. Anti-spectrin IgG was diluted 1:500, anti-band 3 IgG 1:5,000, anti-glycophorin A IgM 1:800, and anti-LWL-1 IgM 1:5. With the exception of LWL-1 and EXP-1 (kind gifts from Kasturi Haldar, Northwestern University, Evanston, IL, ref. 15), all antibodies were purchased from Sigma. Slides were then washed for 1 h and incubated with FITC-conjugated goat anti-mouse IgG or IgM or rhodamine-conjugated goat anti-mouse IgM. Nuclei were stained with Hoechst stain for 5 min at room temperature by using a final concentration of 3 μg/ml in PBS. Samples were viewed by using a Zeiss microscope.

Electron Microscopy. For micrographs of PVM-enclosed merozoite structures (PEMS), synchronous cultures of middle-stage schizonts were treated with E64 for approximately 8 h, washed twice in PBS, and double fixed in 1% gluteraldehyde and 1% osmium tetroxide in 50 mM sodium phosphate buffer for 30 min. Samples were washed in cold H_2O three times and stained with 1% uranyl acetate for 3 h (N. Morrisette, personal communication). Control cultures of intact late-stage schizonts were processed identically. Embedding in polybed (Polyscience) and sectioning were performed at the Microscopy Center (Department of Cell Biology and Physiology, Washington University School of Medicine). Micrographs were viewed by using a Zeiss 902 electron microscope with a Rechert–Joung microtome.

Viability Assay. To determine whether merozoites from within the PEMS are viable, a synchronous culture with a parasitemia of 2% middle-stage schizonts was divided into two and (*i*) treated with 10 μM E64 for 8 h until the vast majority of schizonts developed into PEMS, placed into fresh media without E64, and incubated at 37°C for approximately 18 h; or (*ii*) left untreated for 8 h, placed into fresh media, and incubated at 37°C for 18 h. The resulting parasitemias of late ring-stage cultures were compared by Giemsa-stained smears. In both the control and the E64-treated culture, the resulting parasitemia was approximately 10%.

Purification of PEMS. To purify PEMS, cells from E64-treated cultures, typically with a parasitemia of between 5% and 10%, were collected by centrifugation (10 min, 2,000 $\times$ *g*) and then separated on a cushion of 45% (vol/vol) Percoll (Life Technologies) by centrifugation (20 min, 2,500 $\times$ *g*). PEMS were collected from the top of the cushion, pelleted, washed in media, and added to uninfected red blood cells. Alternatively, PEMS were purified from a procedure adapted from Miller *et al.* (16) and Ward *et al.* (17). E64-treated cultures were first subjected to low-speed centrifugation (5 min at 900 $\times$ *g*) to pellet intact late schizonts and uninfected cells. The supernatant was then centrifuged at high speed (10 min at 1,900 $\times$ *g*) to pellet the PEMS. The pellet was then washed once in RPMI to remove E64, and the PEMS were added to uninfected cells. Results were monitored by Giemsa-stained smears.

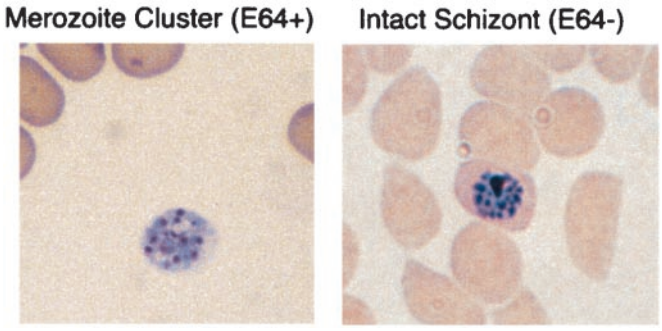

Fig. 1. Effect of E64 treatment on intraerythrocytic schizonts. Synchronous cultures containing middle-stage schizonts were cultured in the presence of 10 μM E64 for approximately 8 h (*Left*). Results were visualized by light microscopy of Giemsa-stained blood smears. An untreated culture of late-stage schizonts is provided for comparison (*Right*).

Results

Effect of the Protease Inhibitor, E64, on Rupture. To better understand the events of rupture and to determine whether the cysteine protease inhibitor E64 affects the process, synchronous HB3 cultures of middle-stage schizonts were treated with 10 μM E64 for approximately 8 h (see *Materials and Methods*). Giemsa-

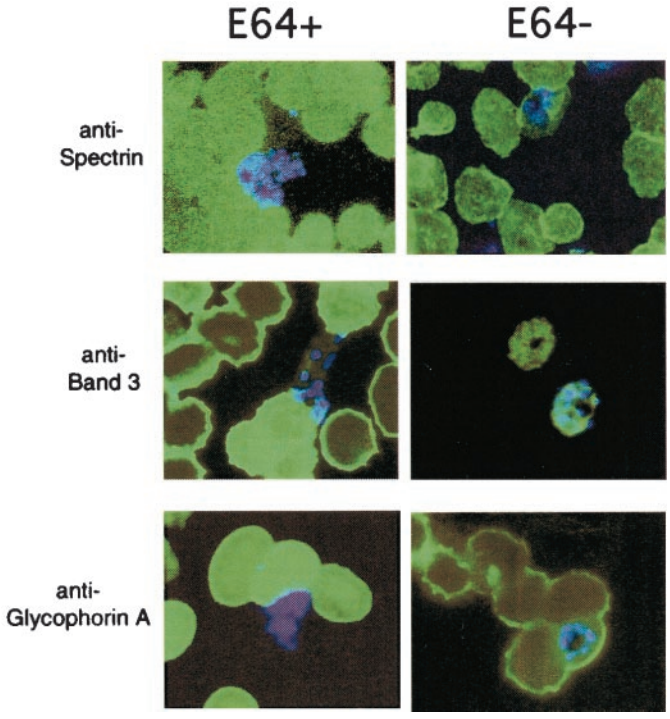

Fig. 2. Localization of erythrocytic proteins and merozoites in schizont-containing blood smears cultured in the presence or absence of E64. Synchronous cultures of middle-stage schizonts were cultured in the presence of 10 μM E64 for approximately 8 h (E64+) or served as untreated controls (E64−). Blood smears of resulting cultures were reacted with monoclonal antibodies directed against spectrin (IgG diluted 1:500), band 3 (IgG diluted 1:5,000), and glycophorin A (IgM diluted 1:800) and then reacted with secondary antibodies FITC-conjugated goat anti-mouse IgG or IgM. Nuclear staining of merozoites was detected by Hoechst stain. Resulting images were visualized by using a Zeiss microscope and merged to compare nuclear stain versus antibody localization patterns.

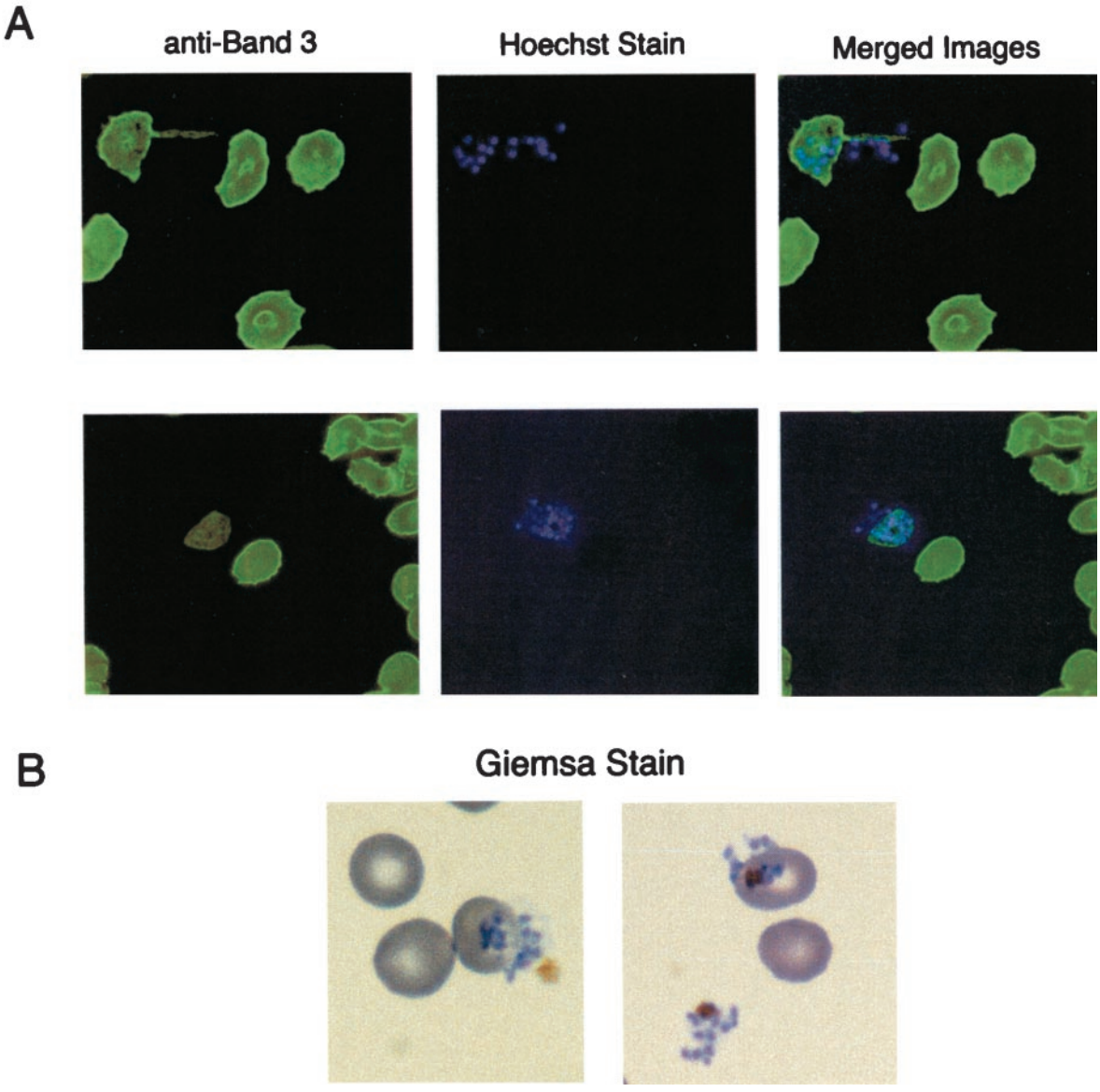

Fig. 3. Analysis of E64-treated parasites in the process of exiting the host cell. (*A*) Blood smears were reacted with antibody directed against band 3 (1:5,000) and stained with Hoescht stain to detect nuclei of merozoites. Resulting images were visualized by using a Zeiss microscope and merged to compare localization patterns. (*B*) In parallel, blood smears were Giemsa-stained and viewed by light microscopy.

stained smears revealed an abundance of merozoite clusters delimited by a thin, transparent membrane, as shown in Fig. 1. These structures contained morphologically normal merozoites loosely associated with hemozoin particles. Nearly all parasites developed into merozoite clusters; few late schizonts and virtually no early rings were evident. Treatment of cultures with 1 μM E64 caused approximately 50% of schizonts to develop into merozoite clusters, and some early rings could be detected. In contrast, the untreated control culture contained mostly early rings with a few late schizonts (not shown). To assess whether the clustering morphology was a product of fixation, unfixed and unstained E64-treated parasites were examined. When viewed by light microscopy, clusters of merozoites could be detected with a morphology clearly distinct from that of late schizonts and free merozoites (data not shown). Addition of E64 to 3D7, W2, and Dd2 cultures gave identical results, indicating that the effect is not strain-specific. These data suggest that the protease inhibitor E64 alters the normal rupturing process, causing an accumulation of merozoites within a thin membrane.

Surprisingly, it was noted that merozoite clusters identical in appearance to those from E64-treated cultures could be detected in untreated cultures, albeit at very low frequency. This finding suggests that the addition of E64 causes an accumulation of intermediates that exist transiently in untreated cultures.

Localization of Red Cell Membrane Proteins Compared with E64-Treated Parasites. To determine whether the membrane surrounding the merozoite clusters is of erythrocytic origin, smears of treated and untreated cultures were examined by indirect immunofluorescence assay using antibodies directed against proteins associated with the erythrocytic membrane. Antibodies directed against glycophorin A and band 3, integral membrane components, and spectrin, a cytoskeletal protein attached to the membrane via interactions with peripheral membrane proteins, were used. Nuclear staining with Hoechst stain demarcated merozoites. As shown in Fig. 2, Hoechst-stained merozoites in E64-treated cultures failed to colocalize with antibodies directed against proteins of the red cell surface. In contrast, nuclear

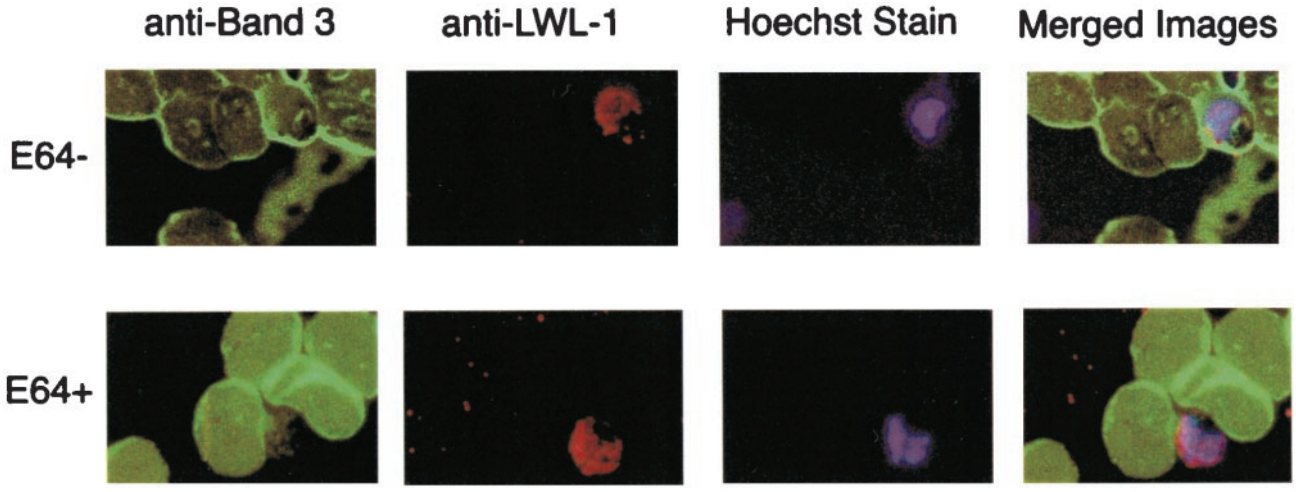

Fig. 4. PVM staining is extraerythrocytic in parasites treated with E64. Erythrocytes containing synchronous cultures of middle-stage schizonts were cultured in the presence of E64 for 8 h (E64+) or served as untreated controls (E64−). Blood smears were reacted with primary antibodies against the erythrocyte membrane marker band 3 (IgG 1:5,000) and the PVM marker LWL-1 (IgM 1:5) and then reacted with secondary antibodies FITC-conjugated goat anti-mouse IgG and rhodamine-conjugated goat anti-mouse IgM. Nuclei of merozoites were detected by using Hoechst stain. Resulting images were viewed by using a Zeiss microscope and merged to compare localization patterns.

staining of merozoites from control cultures localized within the membrane periphery, delineated by reaction with anti-spectrin, anti-band 3, and anti-glycophorin antibodies.

It is also noteworthy that occasionally, Hoechst-stained merozoites from a single cell colocalized with antibodies directed against constituents of the red cell whereas other merozoites were located beyond the periphery of the cell. As shown in Fig. 3, merozoites appeared partially extruded from the membrane of the red cell, as defined with anti-band 3 antibody. Similar results were obtained from red cells defined with anti-spectrin and anti-glycophorin antibodies (data not shown). As depicted in Fig. 3*B*, partial release of the clusters from red cells could also be observed in Giemsa-stained smears. In these circumstances, the merozoite clusters were partially contained within the red blood cell and partially within a thin membrane distinct from that of the red cell. Taken together, these observations suggest that the membrane surrounding the merozoite clusters is distinct from the erythrocytic membrane.

Determination of Membrane Origin. Because the membrane appeared to be emanating from the red cell, it was of interest to determine whether the membrane is derived from the PVM. To this end, smears of E64-treated cultures were Hoechst stained, reacted with anti-band 3 antibody, and reacted with anti-LWL-1 antibody, a well-characterized reagent that reacts with the PVM and its extension, the tubovesicular network (15). As shown in Fig. 4, in smears of E64-treated cultures, LWL-1 antibody colocalized with the Hoechst stain but failed to localize with anti-band 3 antibody. More specifically, the PVM-associated antibodies appeared to surround the extracellular merozoites. This is in marked contrast to localization of LWL-1 antibody in untreated cultures, in which anti-band 3 and LWL-1 antibodies colocalize with the Hoechst stain. Antibody against another PVM marker, EXP-1, gave similar results (not shown). These observations suggest that in E64-treated cultures, the extracellular merozoites are enclosed within a membrane derived from the PVM, clusters termed PEMS.

Electron Micrographs of E64-Treated Parasites. To gain an ultrastructural view of merozoite clusters, E64-treated (Fig. 5 *A*, *C*, and *D*) and untreated (Fig. 5*B*) cultures were visualized by electron microscopy. Similar to the appearance of clusters in Giemsa-stained smears (Fig. 1), merozoites clusters from E64-treated cultures were clearly contained within a single membrane. Often, this membrane appeared as a circular ring encasing the merozoites and was completely intact (Fig. 5 *C* and *D*), but irregular membranous structures similar to that shown in *A* could also be detected. In contrast, merozoites in untreated cultures were contained within two membranes, as is expected of intact intraerythrocytic schizonts. It is also noteworthy that while the merozoites in the E64-treated samples appeared morphologically normal as compared with untreated samples, spacing between merozoites in the clusters appeared less constrained than in intact schizonts.

Viability of Merozoites from PVM-Enclosed Merozoite Structures. Although merozoites within the PEMS appear morphologically normal by both light and electron microscopy, it was of interest to determine whether they are infectious and viable. To this end, a synchronous culture with a parasitemia of 2% was treated with E64 until the vast majority of schizonts developed into PEMS, at which point the E64 was removed. In parallel, untreated schizonts from the same culture, also with a parasitemia of 2%, were allowed to progress. Percent parasitemias of the resulting late-ring stage cultures were evaluated from Giemsa-stained smears of infected erythrocytes. Both treated and untreated cultures had virtually identical levels of parasitemia of approximately 10%; however, the E64-treated culture was delayed in development by approximately 1 h. Both cultures continued to progress normally through several 48-h life cycles (as measured by evaluation of blood smears), suggesting that (*i*) addition of E64 to middle-stage schizonts does not affect development of merozoites within schizonts and (*ii*) after E64 removal, the merozoites from PEMS are infectious and viable.

Purification of PVM-Enclosed Merozoite Structures. Given the fact that E64-mediated PEMS formation is an efficient process and that it yields viable, invasive organisms, it was of interest to purify the structures as a source of merozoites. To this end, synchronous cultures of middle-stage schizonts were treated with E64 for approximately 8 h and purified on a 45% Percoll cushion or, alternatively, were purified by differential centrifugation (see *Materials and Methods*) (16, 17). Both purification procedures gave comparable results. As shown in Fig. 6*A*, large numbers of

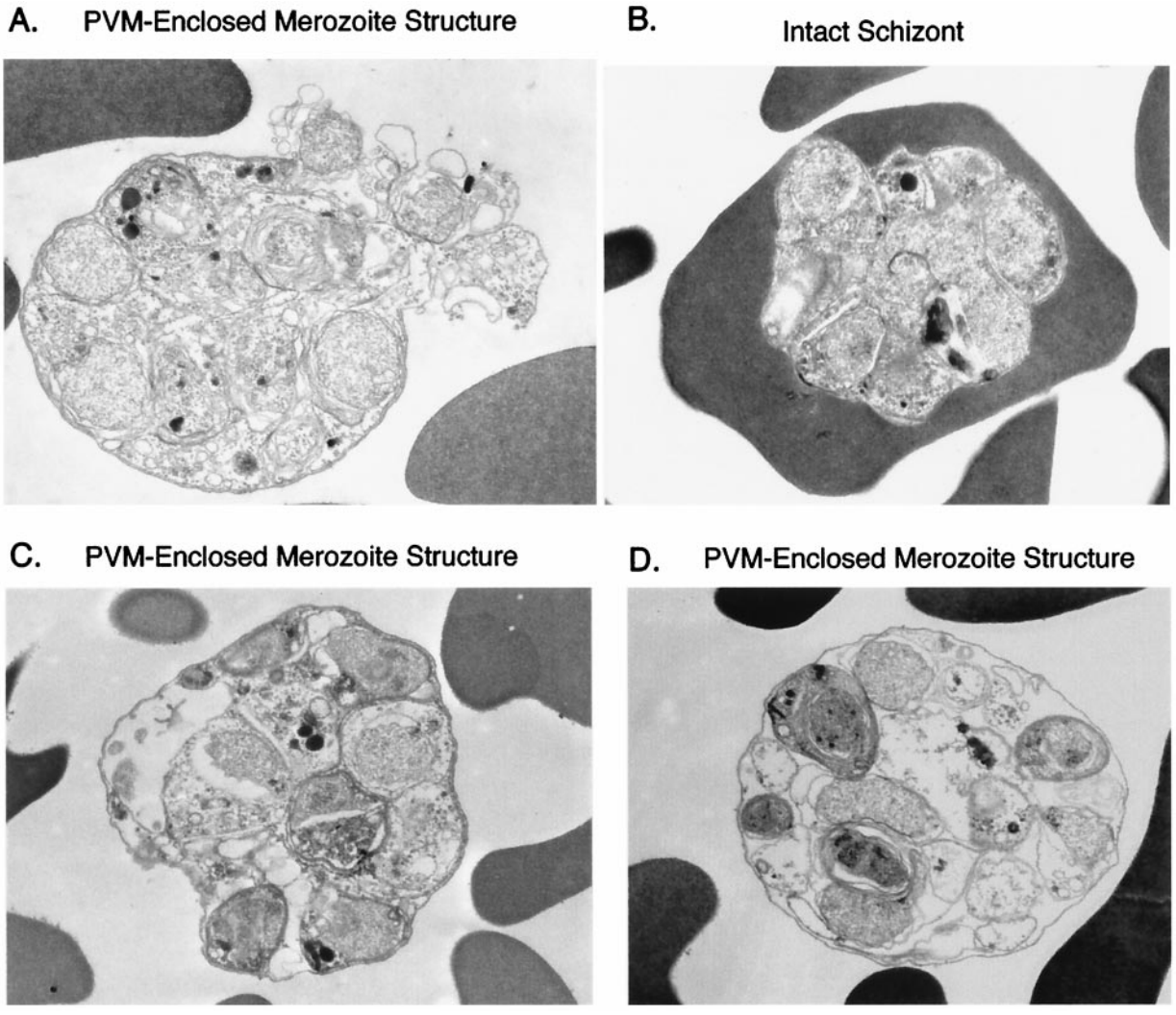

Fig. 5. Electron microscopy analysis of the effect of E64 on schizont maturation. Synchronous cultures of middle-stage schizonts were cultured in the presence of 10 μM E64 for approximately 8 h (*A*, *C*, and *D*), or late-stage schizonts were harvested to serve as a control (*B*). Micrographs were viewed by using a Zeiss 902 electron microscope.

PEMS could be purified entirely devoid of contaminating uninfected red cells and intact late schizonts.

Given the observation that clusters resembling PEMS could be detected, albeit at low frequency, in normal cultures of late schizonts and early rings, it was of interest to determine whether such structures could be purified. To this end, cultures of late-stage schizonts and early rings were processed exactly as E64-treated cultures, as described above. Examination of blood smears after purification revealed structures identical to PEMS, although with approximately 10- to 100-fold lower yields de-

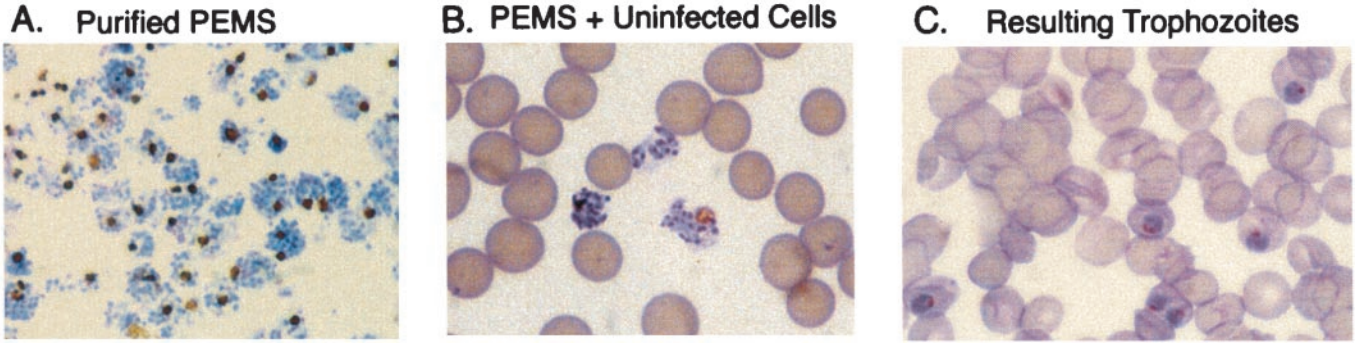

Fig. 6. Giemsa-stained smears of purified PEMS, PEMS added to uninfected erythrocytes, and resulting trophozoites. PEMS were allowed to accumulate in E64-treated cultures of schizonts. Resulting PEMS were purified on a Percoll cushion and added to uninfected erythrocytes. Blood smears 36 h postinfection were stained with Giemsa and viewed by light microscopy.

pending on the experiment, presumably due to a lower frequency in untreated cultures (data not shown).

To assess whether merozoites within purified PEMS are capable of invasion and subsequent development, the structures were purified from E64-treated cultures, washed once in RPMI to remove any residual E64, added to uninfected erythrocytes, and cultured (Fig. 6*B*). Results were monitored by blood smears taken approximately 36 h postinfection. Smears contained tightly synchronous mid-trophozoites (Fig. 6*C*) with a frequency of approximately one to five trophozoites for each PEMS added to the culture. Taken together, these data suggest that merozoites from PEMS are (*i*) invasive, (*ii*) viable and capable of normal development, and (*iii*) establish a simultaneous wave of infection that results in marked synchrony.

Discussion

The events of rupture remain largely uncharacterized, yet evidence suggests that proteases are involved (18). Discovering proteases involved in essential parasite developmental pathways is of particular interest because protease inhibitors have been successfully used in combating infectious diseases, HIV in particular. In this study, we have observed that the cysteine protease inhibitor, E64, affects rupture, and we have identified a previously unknown step in the process.

We show here that when a moderate concentration of the cysteine protease inhibitor, E64, is added to middle-stage schizonts, the normal process of rupture is abrogated and abundant clusters of merozoites accumulate. The merozoites appear morphologically normal but are locked within a thin transparent membrane that is recognized by antibodies to PVM components. These findings differ from studies by Lyon and Haynes (2) in which purified schizonts failed to rupture in the presence of leupeptin or chymostatin. Those authors reported that the membrane surrounding the clusters is of erythrocytic origin, a finding based on indirect immunofluorescence assay using polyclonal antiserum directed against human erythrocytes. Whereas these results suggest that leupeptin and chymostatin inhibit merozoite rupture from the red cell, it is possible that the membrane identified by Lyon and Haynes is derived from the PVM, as it cannot be ruled out that the antibodies directed against whole erythrocytes cross-reacted with proteins on the PVM.

Our data suggest that E64 inhibits a protease involved in release of merozoites from within the PVM. The target protease(s) could be directly involved or could play a role by activating release components (e.g., a phospholipase). Upon removal of E64, the parasites are capable of release from PEMS and can establish normal infections. These results are somewhat surprising given that E64 is an irreversible inhibitor; however, we speculate that a very small amount of protease is necessary for release from the PVM and that, upon E64 removal, enough new protease is synthesized to achieve rupture. The necessity for additional protein synthesis may account for the 1-h delay in development observed after addition of PEMS to uninfected cells. Understanding the exact mechanism and the particular protease/proteases involved, however, will require further study.

Importantly, structures identical in appearance to E64-mediated PEMS can occasionally be detected in untreated control cultures. This correlates with a study by Winograd *et al.* (12), in which video microscopy was used to view events of rupture. In the study, it was noted that merozoites were released together with a residual body containing hemozoin. Membranes could not be identified in their experiments. Based on these observations, we propose that E64 causes an accumulation of an intermediate normally present during the process of rupture. Thus, during a normal infection, merozoites within the PVM exit from the erythrocyte and then rupture rapidly from within the parasite-derived membrane, an event perhaps triggered by exposure to the extracellular milieu.

It is also of interest that the PEMS can be purified easily and efficiently. Up until this point, it has been difficult to separate the PVM from erythrocytic membranes, and as a result, little is known about its macromolecular composition. It is likely that PEMS can be used as a biochemical tool for study of both the PVM and merozoites. In addition, infection of cells with merozoites from purified PEMS establishes a synchronous wave of infection; thus, PEMS can be used to collect large numbers of merozoites to study events of invasion and early parasite development.

We thank C. B. Mamoun and I. Gluzman for insightful discussions. Kasturi Haldar generously provided the LWL-1 and EXP-1 antibodies. We also thank Naomi Morrisette for helpful suggestions regarding electron microscopy and Lori LaRose for technical assistance. D.E.G. is a recipient of the Burroughs Wellcome Fund Scholar Award in Molecular Parasitology.

1. Trigg, P. I. & Kondrachine, A. V. (1998) in *Malaria: Parasite Biology, Pathogenesis and Protection*, ed. Sherman, I. W. (Am. Soc. Microbiol., Washington, DC), pp. 11–22.
2. Lyon, A. J. & Haynes, J. D. (1986) *J. Immunol.* **136,** 2245–2251.
3. Rosenthal, P. J., Kim, K., McKerrow, J. H. & Leech, J. H. (1987) *J. Exp. Med.* **166,** 816–821.
4. Delplace, P., Dubremetz, J. F., Fortier, B. & Vernes, A. (1985) *Mol. Biochem. Parasitol.* **17,** 239–251.
5. Bzik, D. J., Li, W. B., Horii, T. & Inselburg, J. (1988) *Mol. Biochem. Parasitol.* **30,** 279–288.
6. Knapp, B., Hundt, E., Nau, U. & Kupper, H. A. (1989) *Mol. Biochem. Parasitol.* **32,** 73–84.
7. Gor, D. O., Li, A. C., Wiser, M. F. & Rosenthal, P. J. (1998) *Mol. Biochem. Parasitol.* **95,** 153–158.
8. Higgins, D. G., McConnell, D. J. & Sharp, P. M. (1989) *Nature (London)* **340,** 604.
9. Delplace, P., Fortier, B., Tronchin, G., Dubremetz, J. F. & Vernes, A. (1987) *Mol. Biochem. Parasitol.* **23,** 193–201.
10. Debrabant, A. & Delplace, P. (1989) *Mol. Biochem. Parasitol.* **33,** 151–158.
11. Le Bonniec, S., Deregnaucourt, C., Redeker, V., Banerjee, R., Grellier, P., Goldberg, D. E. & Schrevel, J. (1999) *J. Biol. Chem.* **274,** 14218–14223.
12. Winograd, E., Clavijo, C. A., Bustamante, L. Y. & Jaramillo, M. (1999) *Parasitol. Res.* **85,** 621–624.
13. Trager, W. & Jensen, J. B. (1976) *Science* **193,** 673–675.
14. Lambros, C. & Vanderberg, J. P. (1979) *J. Parasitol.* **65,** 418–420.
15. Li, W., Das, A., Song, J., Crary, J. L. & Haldar, K. (1991) *Mol. Biochem. Parasitol.* **49,** 157–168.
16. Miller, L. H., Hudson, D., Rener, J. & Taylor, D. (1983) *J. Clin. Invest.* **72,** 1357–1364.
17. Ward, G. E., Miller, L. H. & Dvorak, J. A. (1993) *J. Cell Sci.* **106,** 237–248.
18. McKerrow, J. H., Sun, E., Rosenthal, P. J. & Bouvier, J. (1993) *Annu. Rev. Microbiol.* **47,** 821–853.