dium pentobarbital and perfused through a heart puncture with saline and then with 10 percent buffered formalin. Frozen sections of the hypothalamus were cut at 40 μm and stained with cresyl violet.
12. All insulin injections were given intraperitoneally in saline in a volume equal to 0.012 cm^3 per gram of body weight. The insulin used was U-40 beef and pork insulin (Lilly).
13. The statistical methods employed were two-way analysis of variance and Tukey's test.
14. Blood glucose content was determined on tail vein samples by using the fluorometric procedure of O. H. Lowry, J. V. Passonneau, F. X. Hasselberger, and D. W. Schultz [*J. Biol. Chem.* **239**, 18 (1964)].
15. D. E. Wooley and P. S. Timiras, *Am. J. Physiol.* **202**, 379 (1962).
16. J. A. Ferrendelli and D. B. McDougal, Jr., *J. Neurochem.* **18**, 1197 (1971).
17. ———, *ibid.*, p. 1207.
18. L. J. King, O. H. Lowry, J. V. Passonneau, V. Veason, *ibid.* **14**, 559 (1967).
19. M. Tarr, D. Brada, F. E. Samson, Jr., *Am. J. Physiol.* **203**, 690 (1962).
20. G. C. Tamasi and E. J. Drenick, *Endocrinology* **92**, 1277 (1973).
21. Supported in part by NIH training grant GM 01417.
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Invasion of Erythrocytes by Malaria Merozoites

Abstract. *An electro-optical system was developed to record microscope images with high resolution at low light intensities. The system was used to study the invasion of erythrocytes by malaria merozoites. Invasion consists of attachment of the anterior end of the parasite to the erythrocyte, deformation of the erythrocyte, and entry of the parasite by erythrocyte membrane invagination.*

The events that occur during the erythrocytic stage of a malaria infection have long been known. For example, the rupture of an infected erythrocyte results in the liberation of individual parasites (merozoites) which infect other erythrocytes. This is a particularly crucial sequence of events, for if the penetration of erythrocytes by merozoites could be interrupted, the erythrocytic cycle of the parasite would be broken and infection terminated.

Observations of the invasion of erythrocytes by merozoites have been reported (*1*). However, the problems of maintaining host cells and parasites under optimum physiologic conditions and observing and recording their interactions under optimum optical conditions have limited the extent of these studies. In this report we present a method for overcoming these problems and describe events that occur during the rupture of schizont-infected erythrocytes and the subsequent invasion of other erythrocytes by merozoites. A hypothesis is presented to explain some of the phenomena observed.

Erythrocytes containing mature schizonts of *Plasmodium knowlesi* were transferred from infected monkeys to culture medium, mixed with uninfected erythrocytes (*2*), and inoculated into Dvorak-Stotler controlled-environment culture chambers (*3*). After the erythrocytes had settled onto the lower cover glass, the chambers were continuously perfused with fresh culture medium and maintained at a temperature of 35° ± 0.2°C (*4*).

Microscopy was performed with an inverted microscope (Leitz Diavert) equipped with Smith differential interference optics. The image from the microscope was projected onto the faceplate of an RCA type 8673 image orthicon installed in a high-gain video camera suitable for the requisite operation at low light intensities. The resulting video signal was processed by an image enhancer (*5*) and recorded on 1-inch video tape for storage. The video signal from selected tape segments was processed by a time base corrector (*6*) and subsequently used to produce a 16-mm motion picture negative by electron beam recording (*7*).

The rupture of schizont-infected erythrocytes is always preceded by the coalescence of malaria pigment into a single unit within a residual body (Fig. 1a). This is followed by vesiculation of the erythrocyte membrane (Fig. 1b), distortion of the erythrocyte due to movement of intracellular merozoites (Fig. 1c), and finally swelling (Fig. 1d) and rupture of the erythrocyte (Fig. 1e) and release of the merozoites with explosive suddenness (Fig. 1f). Erythrocyte swelling begins about 9 seconds before rupture and results in a net increase in volume of approximately 20 percent. Rupture of the infected erythrocyte occurs within 1 minute after the first appearance of erythrocyte membrane vesiculation. Occasionally, merozoites remain attached to the residual body. Aside from Brownian motion, individual merozoites which settle onto the lower cover glass of the chamber display pivotal motion, bending, or generalized contraction with a variable point of contact to the glass; these movements are random relative to surrounding erythrocytes.

Penetration of an erythrocyte occurs only if the anterior end of the merozite, containing the paired organelles, contacts the erythrocyte (see cover, upper left photograph; 0 second). The initial attachment between the anterior end of the merozoite and an erythrocyte results in a rapid and marked deformation of the erythrocyte for a period of 5 to 10 seconds (cover, left to right, photographs 2 to 6; 0.4, 1.3, 1.5, 5.2, and 6.6 seconds). Deformation

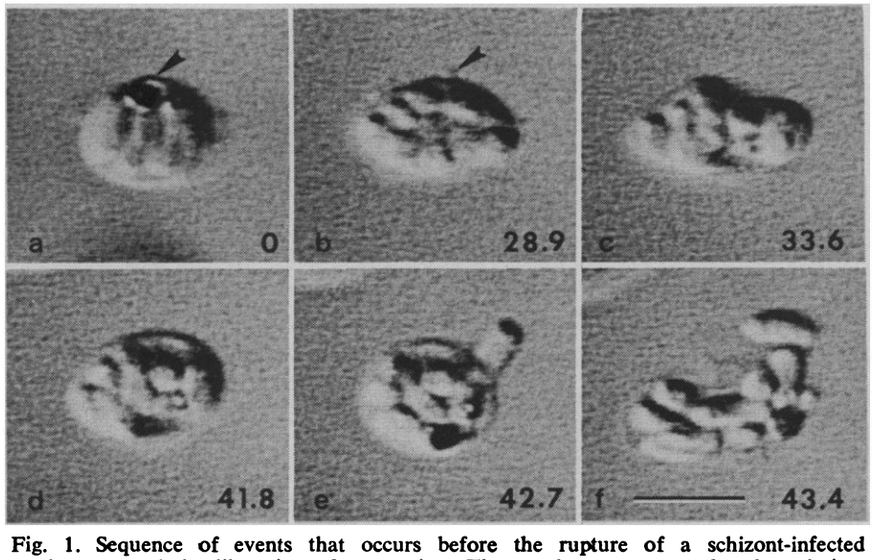

Fig. 1. Sequence of events that occurs before the rupture of a schizont-infected erythrocyte and the liberation of merozoites. The numbers represent the elapsed time in seconds. Scale marker, 5 μm.

occurs irrespective of the site on the erythrocyte contacted by the anterior end of the merozoite. The merozoite remains attached to the erythrocyte but does not enter the cell during this initial deformation (cover, photograph 7; 8.2 seconds). The actual interiorization of a merozoite requires approximately 10 to 20 seconds for completion (cover, photographs 8 to 11; 9.7, 21.8, 25.3, and 28 seconds). Following this interiorization, the erythrocyte is again deformed (cover, photograph 12; 35.3 seconds). This second wave of deformation continues intermittently for 10 to 15 minutes, after which the parasite becomes quiescent and the erythrocyte resumes its biconcave shape. Alternately, the infected erythrocyte may crenate and subsequently swell and become a spherocyte. This process is accompanied by rapid spinning of the merozoite in a discrete, localized area within the developing spherocyte. Swelling may continue until lysis of the spherocyte occurs, or the cell may gradually return to a normal biconcave shape. However, erythrocytes always lyse after simultaneous invasion by more than two merozoites or by a merozoite that retains its posterior attachment to a residual body. The paired organelles of merozoites are still present after complete interiorization of the parasites. This is most readily seen in infected erythrocytes that have lysed.

The ability to critically observe and continuously record the rupture of schizont-infected erythrocytes and the subsequent penetration of other erythrocytes under optimum physiological and optical conditions has clarified certain aspects of this host-parasite interaction, but raised several new questions. First, this study has confirmed the definite orientation dependence of merozoites during penetration of erythrocytes. The anterior end of the merozoite, containing the paired organelles, must contact the erythrocyte before the actual penetration phase is initiated. Second, penetration is a stepwise phenomenon. It consists initially of an attachment phase accompanied by deformation of the erythrocyte. This is followed by a relatively slow interiorization of the parasite and then another wave of deformation of the erythrocyte. Finally, the paired organelles, which may play a role in attachment or interiorization of the merozoite, are not visibly altered after the parasite is within the erythrocyte.

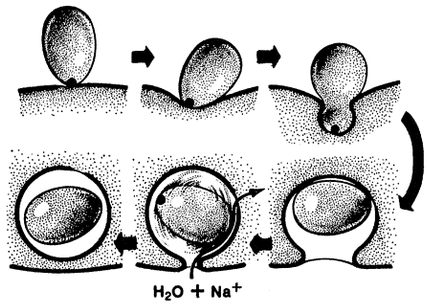

Fig. 2. Schematic representation of the penetration of an erythrocyte by a malaria merozoite.

The major questions arising from this study concern the actual mechanisms involved in erythrocyte deformation and parasite interiorization. Severe erythrocyte deformation occurs twice—after initial attachment of the merozoite and again after complete interiorization of the parasite. These changes are probably induced by the merozoite, but neither the site or sites affected in the erythrocyte (such as the membrane or hemoglobin) nor the physical and chemical nature of the initial membrane lesion is known. The actual interiorization of the parasite may be an active process on the part of the host cell or parasite. Some investigators believe protozoans of the subphylum Sporozoa are taken into the host cells by endocytosis (*8*). However, recent studies suggest an alternate hypothesis, that the interiorization of these parasites into "nonphagocytic" cells is an active process on the part of the parasite (*9*).

Based on the data available to date, a theoretical model for the penetration of erythrocytes by malaria merozoites can be devised (Fig. 2). Free merozoites come in contact with erythrocytes by chance. Properly oriented contact results in the attachment of merozoites to erythrocytes through an interaction between receptor sites on the anterior end of the merozoite and the erythrocyte surface (*2*). A localized lesion may be produced in the erythrocyte membrane at the site of attachment. Widespread deformation of the erythrocyte could occur as a result of this lesion. After this deformation has subsided, the merozoite enters the erythrocyte by producing a localized invagination of the erythrocyte membrane. Although within the erythrocyte, the parasite is still exposed to the external milieu through the orifice of the resulting invagination. The orifice and membrane lesion permit sodium, water, and possibly calcium to enter the erythrocyte at a rate in excess of its normal exchange capacity. However, the lesion is too small to permit hemoglobin or other large molecules to leave the erythrocyte. Consequently, the erythrocyte rapidly spurs and swells. A mechanism by which the membrane lesion could be isolated from the external milieu is fusion of the erythrocyte membrane at the orifice of the invagination (*10*). After fusion, the normal intracellular water and electrolyte content of the erythrocyte is restored and the cell serves as a host for development of the malaria parasite.

Two morphologic observations provide evidence for a discrete membrane lesion within the invagination and a fusion of the erythrocyte membrane at the orifice of the invagination. First, the spinning of a merozoite within the invagination is associated with erythrocyte swelling; the spinning stops at the time of erythrocyte lysis or fusion of the erythrocyte membrane. Spinning is caused by flow streams created by the passage of fluid through the orifice, around the parasite, and through the membrane lesion. Second, the invasion of erythrocytes by merozoites attached to a residual body always results in erythrocyte lysis. In this case, the orifice remaining after merozoite-produced invagination cannot fuse as it is occluded by the residual body attached to the merozoite.

The data presented here provide a foundation for further studies of the interaction of malaria parasites with vertebrate cells. Equally important, the system developed for the observation and recording of rapidly occurring events under controlled environmental conditions at high resolution and low light intensities can be applied to other areas of biology.

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References and Notes

1. W. Trager, *Trans. R. Soc. Trop. Med. Hyg.* **50**, 419 (1956); C. Huff, A. Pipkin, A. Weathersby, D. Jensen, *J. Biophys. Biochem. Cytol.* **7**, 93 (1960).
2. L. H. Miller, J. Dvorak, T. Shiroishi, J. Durocher, *J. Exp. Med.* **138**, 1597 (1973).
3. J. A. Dvorak and W. F. Stotler, *Exp. Cell Res.* **68**, 144 (1971). The culture chamber is available from Carl Zeiss, Inc., New York.
4. Temperature was maintained with a Nicholson Precision Instruments model C-300 air stream incubator, available from Carl Zeiss, Inc., New York.
5. This step, utilizing a CBS Laboratories model Mark 3 series 8000 enhancer, was required to retain high-frequency information for video tape recording.
6. This step, utilizing a Consolidated Video Systems model 500 time base corrector, was required to provide a stable signal to the electron beam recorder.
7. The transfer to 16-mm film was made with a 3M Corp., Mincom Division, electron beam recorder. A 16-mm motion picture depicting all of the events described in this report is available for distribution from the authors.
8. R. Ladda, M. Aikawa, H. Sprinz, *J. Parasitol.* **55**, 633 (1969); W. Trager, *Science* **183**, 269 (1974); T. C. Jones, S. Yeh, J. Hirsch, *J. Exp. Med.* **136**, 1157 (1972).
9. J. B. Jensen and D. M. Hammond, *J. Parasitol.* **50**, 35 (abstract) (1974).
10. W. T. Mason and Y. F. Lee, *Nat. New Biol.* **244**, 143 (1973).

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Selection of a Variant Lymphoma Cell Deficient in Adenylate Cyclase

Abstract. *Isoproterenol, a stimulator of adenylate cyclase, was used to select a stable variant clone of mouse lymphosarcoma cells deficient in the enzyme. The inability of four different stimulators to activate cyclic adenosine monophosphate synthesis in the variant, in contrast to its wild-type parent, implies that in normal cells one type of adenylate cyclase molecule can respond to different activators.*

The earliest detectable effect of many hormones on their target tissues is the activation of adenylate cyclase, which catalyzes the formation of adenosine 3′,5′-monophosphate (cyclic AMP) from adenosine triphosphate. Because the enzyme is rather labile, bound to membranes, and has resisted purification, most knowledge of this crucial step in hormone action derives from experiments with particulate fractions of broken cells (*1*). Cyclic AMP synthesis in such preparations is often stimulated by more than one hormone, and apparently always by fluoride ion as well.

Since maximally effective concentrations of two or more different agents usually fail to produce additive enzyme stimulation (*2*), it is believed that only one kind of adenylate cyclase molecule is required for a cell to respond to multiple stimuli; alternatively, an individual cell might conceivably possess a panoply of different enzymes, each specifically responsive to a different hormone. Such a question cannot be answered rigorously without extensive purification and separation of adenylate cyclase or cyclases and other components of membranes, followed by their functional recombination in vitro —a daunting task.

We report here a genetic approach to this question, which takes advantage of a cultured cell line that is killed by prolonged elevation of intracellular cyclic AMP. We have selected stable variant clones of cells that are resistant to the cytolytic effect of isoproterenol, a stimulator of adenylate cyclase. In one such clone, in contrast to the parental cell line, cyclic AMP formation cannot be stimulated by isoproterenol or three other stimulators, including fluoride. The simplest explanation of this result, a single mutation involving adenylate cyclase, implies that in normal cells one type of enzyme molecule can respond to different stimulators of cyclic AMP synthesis.

S49 mouse lymphosarcoma cells in culture are killed by exposure for 48 to 72 hours to N^6,2′-*O*-dibutyryl (DB) cyclic AMP or to agents that stimulate the synthesis of endogenous cyclic AMP (*3*). In order to select and isolate the adenylate cyclase–deficient variant cell line, wild-type S49 cells were cloned in soft agar overlying a fibroblast feeder layer (*4, 5*). Cloning efficiency in the absence of cytocidal drugs was 50 to 90 percent, but was reduced to 0.15 percent when the agar

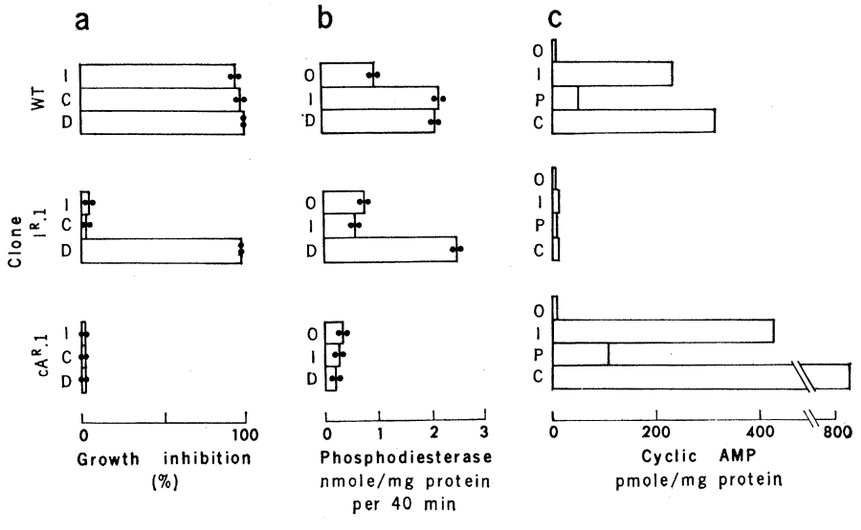

Fig. 1. Comparison of the phenotype of wild-type cells (WT, top) with those of isoproterenol resistant (I^R.1, middle) and cyclic AMP resistant clones (cA^R.1, bottom). (a) Growth inhibition by isoproterenol (I) (1.0 μM), cholera toxin (C) (30 ng/ml), or DB cyclic AMP (D) (0.2 mM). Cells were suspended at a concentration of 2×10^5 ml^{-1} in growth medium containing effectors plus RO 20-1724 (30 μM), and viable cells (those excluding trypan blue) were counted 72 hours later. Results are expressed as the percentage inhibition of growth by the effector (in duplicate incubations) as compared with cell growth in control incubations containing RO 20-1724 alone, in which cells multiplied to densities of 2.1×10^6 to 2.4×10^6 ml^{-1}. (b) Induction of cyclic AMP phosphodiesterase. Logarithmically growing cells were untreated (O) or given isoproterenol (I) (1.0 μM), or DB cyclic AMP (D) (0.2 mM) for 4.5 hours, centrifuged, sonicated, and assayed for phosphodiesterase activity as described (*3, 8, 17*) at a substrate (cyclic AMP) concentration of 1.0 μM. Each bar represents the mean of duplicate incubations. (c) Cyclic AMP accumulation in logarithmically growing untreated cells (O) or cells exposed to isoproterenol (I) (1.0 μM), PGE_1 (P) (0.1 μM), or cholera toxin (C) (30 ng/ml). Cells were incubated with cholera toxin for 90 minutes and with the other agents for 15 minutes before being centrifuged and assayed for cyclic AMP as described (*3, 8*) by the competition binding assay of Gilman (*11*). Purified cholera toxin, prepared by Finkelstein and Lospalluto (*18*), was obtained from Dr. John Seal, NIH Standards. Prostaglandin E_1 was a gift from Dr. John Pike of the Upjohn Company. Other agents were obtained commercially.