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INVOLVEMENT OF MALARIAL PROTEASES IN THE INTERACTION BETWEEN THE PARASITE AND HOST ERYTHROCYTE IN *PLASMODIUM KNOWLESI* INFECTIONS

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ABSTRACT: The effect of protease inhibitors obtained from the culture filtrates of actinomycetes (pepstatin, chymostatin, leupeptin, phosphoramidon and elastatinal) on the in vitro invasion of erythrocytes from rhesus and assamese monkeys by *Plasmodium knowlesi* merozoites was studied. The presence of these inhibitors had no effect on release of merozoites from schizonts but inhibited entry of the parasite into the red cell. Thus, at 50 μg/ml, chymostatin and leupeptin completely blocked the invasion whereas pepstatin and elastatinal showed 50% inhibition. Phosphoramidon at this concentration gave only 30% inhibition. Pretreatment of the erythrocytes with these inhibitors did not block invasion. Also, the intracellular development of the parasite was totally unaffected in the presence of these agents. These results suggested that proteases of merozoites might play some crucial role in the invasion process.

Invasion of erythrocytes by malarial merozoites is initiated by attachment of the apical end of the merozoite to a specific region of the host cell surface, creating a small depression in the erythrocyte membrane (Ladda et al., 1969; Aikawa et al., 1978; McLaren et al., 1979). The area of the erythrocyte membrane to which the merozoite is attached becomes thickened and forms a junction with the plasma membrane of the merozoite, which moves along the confronted membranes as invasion progresses (Aikawa et al., 1978).

The extracellular merozoite is covered with a prominent surface coat (Ladda et al., 1969), but, during invasion, this coat appears to be absent from the portion of the merozoite within the erythrocyte invagination (Ladda et al., 1969; Aikawa et al., 1978). The intramembranous particles of the invaginating vacuolar membranes progressively disappear, so that by the time the invading merozoite is completely enclosed, the vacuolar membrane is virtually devoid of trans-membrane proteins and is, therefore, lipid rich (McLaren et al., 1979). To explain observations such as dissolution of the merozoite's surface coat, formation of moving junctions, and disappearance of intramembranous particles of the invaginating vacuolar membrane during interaction between erythrocytes and merozoites, involvement of the parasite's proteases in the invasion process has been postulated (Ladda et al., 1969; Aikawa et al., 1978; McLaren et al., 1979). However, only Banyal et al. (1978; 1980) have suggested that isolated merozoites possess strong proteolytic enzymes. They recently demonstrated proteases in merozoites of *Plasmodium knowlesi* in vitro. The present study provides evidence for possible involvement of the parasite's proteases in the process of entrance of merozoites into host cells.

MATERIALS AND METHODS

Rhesus monkeys, *Macaca mulatta*, and assamese monkeys, *M. assamensis*, weighing 3 to 8 kg were kept in the light between 0700 hr and 1900 hr to maintain the synchronicity of the parasite. The W_1 strain of *P. knowlesi* was used. Normal and *P. knowlesi*-infected blood samples from monkeys were collected in glucose-citrate under sterile conditions by cardiac or venipuncture and centrifuged at 800 *g* for 10 min. Plasma and the buffy coat were removed and erythrocytes were washed thrice with PBS, pH 7.2. From the infected samples, the brown layer of schizont-infected cells was separated from the underlying erythrocytes. The culture medium used was RPMI-1640 buffered with 3.5% (v/v) of 0.5 M $NaHCO_3$ and supplemented with glucose (2 mg/ml), ATP (0.3 mg/ml), penicillin (100 units/ml), streptomycin (0.1 mg/ml), and HEPES (25 mM). The pH of the medium was adjusted to 7.4 with additional $NaHCO_3$. The medium was sterilized using a membrane filter. Parasites were cultured in 50 ml Erlenmeyer flasks each containing 3.0 ml medium, 0.3 ml inactivated rhesus or assamese monkey serum, 0.1 ml desired concentration of inhibitor, and 0.25 ml packed erythrocytes (normal and schizont-infected erythrocytes were mixed in such a proportion that initial parasitaemia did not exceed 2%). The flasks were incubated at 37 C in 7% CO_2, 1% O_2, and 92% N_2. After 15 hr incubation, the cultures were centrifuged at 1,000 *g* for 10 min, and smears were prepared and stained with Giemsa. No schizonts were left in the medium and the

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TABLE I. *Effect of different inhibitors of proteases on the in vitro invasion of erythrocytes from rhesus and assamese monkeys by* P. knowlesi.

Inhibitor*	Concentration (µg/ml)	% Inhibition†	
		M. mulatta	*M. assamensis*
Pepstatin	5	1.5	2.7
	10	3.9	3.0
	20	17.0	21.5
	50	49.2	51.7
Chymostatin	5	71.5	71.0
	10	84.6	85.0
	20	87.7	97.5
	50	100.0	100.0
Leupeptin	5	26.2	15.0
	10	30.8	31.7
	20	64.6	67.4
	50	98.2	90.0
Phosphoramidon	5	0	1.0
	10	0	1.7
	20	9.3	8.5
	50	32.3	32.0
Elastatinal	5	0	0
	10	0	6.7
	20	9.3	11.0
	50	52.3	49.8

* Pepstatin and chymostatin were dissolved in water containing a very small amount of acetic acid. Control flasks containing acetic acid gave almost the same results as the controls without acetic acid. All other inhibitors were soluble in water.
† Each value is a mean of triplicate experiments. Four- to sixfold multiplication of parasites was observed in controls with both types of erythrocytes.

TABLE II. *In vitro invasion by* P. knowlesi *of erythrocytes pretreated with protease inhibitors.*

Inhibitor (50 µg/ml)	Fold-multiplication of parasites*	
	M. mulatta	*M. assamensis*
Pepstatin	5.12	5.26
	5.00	6.12
	5.35	5.10
Chymostatin	4.71	4.72
	6.20	6.90
	6.10	5.35
Leupeptin	4.52	5.12
	6.53	6.35
	5.73	5.60
Phosphoramidon	4.50	4.69
	6.30	6.80
	4.95	5.50
Elastatinal	4.92	5.12
	6.55	6.35
	5.82	5.20
Control	4.65	4.82
	6.25	6.86
	5.90	5.35

* Incubations were 15 hr in duration.

parasites which entered host cells were seen as early and late trophozoites. Percent inhibition was calculated assuming the number of parasites in the control experiments as 100%.

RESULTS

The effects of different inhibitors on the invasion of erythrocytes by *P. knowlesi* are shown in Table I. Chymostatin, which inhibits chymotrypsin but not trypsin (Umezawa et al., 1970a), completely blocked invasion at 50 µg/ml concentration. Even at the lowest concentration (5 µg/ml) used, it inhibited more than 70%. Leupeptin (50 µg/ml), an inhibitor of trypsin but not of chymotrypsin (Aoyagi et al., 1969), blocked invasion by 90%. More than 60% inhibition of invasion was seen at 20 µg/ml but the inhibition was not as significant as with chymostatin at this concentration. Pepstatin, an inhibitor of cathepsin D (Umezawa et al., 1970b), and elastatinal, an inhibitor of elastase (Umezawa et al., 1973), showed 50% inhibition at 50 µg/ml whereas phosphoramidon, an inhibitor of neutral metalloendopeptidases (Suda et al., 1973), blocked 32% of invasion at the maximum concentration used.

To determine if the presence of these inhibitors in the culture system induced any physiological changes in the host erythrocytes or in the intraerythrocytic parasites the following experiments were done. Erythrocytes from each host were preincubated with the maximum concentration of inhibitors at 37 C for 4 hr. Inhibitor-treated erythrocytes were then washed thrice with phosphate buffer saline, pH 7.2, suspended in the culture medium in the flasks, and then schizont-infected cells were added. Smears prepared after 15 hr incubation showed that such erythrocytes were equally susceptible to *P. knowlesi* as were the control cells (Table II). Erythrocytes infected with a synchronous population of ring-stage *P. knowlesi* were incubated in the presence of inhibitors in the culture system. After 15 hr, trophozoites and multinucleated schizonts were observed in the smears (Table III).

DISCUSSION

These experiments demonstrated that protease-inhibitors used did not exert any effect either on the susceptibility of the host erythrocytes to infection or on the intracellular de-

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TABLE III. *In vitro intracellular development of* P. knowlesi *in the presence of protease inhibitors.*

Inhibitor (50 μg/ml)	Number of parasites/10^4 erythrocytes*					
	M. mulatta			*M. assamensis*		
	R†	T	S	R	T	S
Pepstatin	–	22	298	–	16	286
	–	26	216	–	13	295
	–	9	260	–	35	281
Chymostatin	–	12	352	–	19	318
	–	19	295	–	28	281
	5	36	251	–	32	261
Leupeptin	–	21	302	–	15	321
	–	37	268	–	16	266
	–	32	292	4	27	304
Phosphoramidon	–	23	286	–	10	286
	–	19	216	–	17	315
	–	13	256	–	23	292
Elastatinal	–	19	320	–	16	299
	–	21	285	1	18	283
	2	5	272	–	15	270
Control	–	17	333	–	28	301
	–	27	265	1	10	298
	1	23	288	–	17	284

* Initial inoculum contained ring stages only. The parasitemia was 2.0 to 4.0%. Incubations were carried out for 20 hr.

† R = Ring, T = Trophozoite, S = Schizont.

velopment of the parasite. Moreover, the complete absence of free schizonts in the culture medium containing the inhibitors suggested that these agents had no effect on release of merozoites from RBC's containing schizonts. We propose that merozoites secrete proteases similar to trypsin and chymotrypsin, and that these proteases have some important role in the process of invasion of the red cells.

The role of proteases in invasion of erythrocytes by merozoites could be manifold. Observations such as disappearance of the surface coat, initial formation of a thick junction between the apical end of the merozoite and the erythrocyte membrane, induction of invagination into the erythrocyte's membrane, formation of moving junctions between the merozoite and the erythrocyte membrane, and disappearance of intramembranous particles of the invaginating vacuolar membrane during invasion may all be rationalized by involvement of proteases released by the merozoite during invasion.

The initial specificity for attachment of the merozoite to a specific receptor site present on the erythrocyte's surface appeared to reside in part of the surface coat present on the apical end. This specific attachment may signal release of some proteases together with other unknown factors by the merozoite. Prior to the release of these proteases at the site of contact between merozoite and erythrocyte, the position of the surface coat covering the apical end should be dissolved. These enzymes may now cleave the exposed sites of host cell membrane-bound proteins, thus reducing the mechanical stability of the membrane which in turn should make the membrane more deformable (Egmond et al., 1979). Moreover, if the processed glycoproteins happen to be transmembrane proteins, this may adversely affect control of the erythrocyte's deformability by the cytoskeletal proteins because transmembrane proteins present in human erythrocyte membranes are known to be linked, directly or indirectly, with cytoskeletal proteins of the cell (Bennett and Stenbuck, 1979; Steck, 1974).

The observed absence of intramembranous particles in developing parasitophorous vacuole has been best explained by the postulate that expansion of the vacuolar membrane results from incorporation of merozoite-secreted membrane into the host cell membrane (McLaren et al., 1979). This expansion may require fusion of lipid vesicles (secreted by the merozoite) with the protein-free portion of the host cell membrane (Tyrrell et al., 1976).

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