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Growth of *Plasmodium falciparum* in human erythrocytes containing abnormal membrane proteins

(malaria/cytoskeleton/hereditary spherocytosis/hereditary elliptocytosis/erythrocyte membrane spectrin, protein 4.1, and band 3)

S. SCHULMAN*†, E. F. ROTH, JR.*, B. CHENG*, A. C. RYBICKI*, I. I. SUSSMAN*, M. WONG*, W. WANG‡, H. M. RANNEY§, R. L. NAGEL*, AND R. S. SCHWARTZ*¶

*Division of Hematology, Albert Einstein College of Medicine and Montefiore Medical Center, Bronx, NY 10467; †Department of Natural Sciences, Baruch College, City University of New York, New York, NY 10010; ‡St. Judes Children's Research Hospital, Memphis, TN 38101; and §The University of California at San Diego, San Diego, CA 92093

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ABSTRACT **To evaluate the role of erythrocyte (RBC) membrane proteins in the invasion and maturation of *Plasmodium falciparum*, we have studied, in culture, abnormal RBCs containing quantitative or qualitative membrane protein defects. These defects included hereditary spherocytosis (HS) due to decreases in the content of spectrin [HS(Sp^+)], hereditary elliptocytosis (HE) due to protein 4.1 deficiency [HE(4.1^0)], HE due to a spectrin αI domain structural variant that results in increased content of spectrin dimers [HE($Sp\alpha^{I/65}$)], and band 3 structural variants. Parasite invasion, measured by the initial uptake of [^{3}H]hypoxanthine 18 hr after inoculation with merozoites, was normal in all of the pathologic RBCs. In contrast, RBCs from six HS(Sp^+) subjects showed marked growth inhibition that became apparent after the first or second growth cycle. Preincubation of HS(Sp^+) RBCs in culture for 3 days did not alter these results. Normal parasite growth was observed in RBCs from one HS subject with normal membrane spectrin content. The extent of decreased parasite growth in HS(Sp^+) RBCs closely correlated with the extent of RBC spectrin deficiency ($r = 0.90$). Homogeneous subpopulations of dense HS RBCs exhibited decreased parasite growth to the same extent as did HS whole blood. RBCs from four HE subjects showed marked parasite growth inhibition, the extent of which correlated with the content of spectrin dimers ($r = 0.94$). RBCs from two unrelated subjects with structural variants of band 3 sustained normal parasite growth. Decreased growth in the pathologic RBCs was not the result of decreased ATP or glutathione levels or of increased RBC hemolysis. We conclude that abnormal parasite growth in these RBCs is not the consequence of metabolic or secondary defects. Instead, we suggest that a functionally and structurally normal host membrane is indispensable for parasite growth and development.**

Several erythrocyte (RBC) genetic defects, particularly those with polymorphic gene frequencies, have most likely been expanded in human populations by the selective pressure of *Plasmodium falciparum* malaria (1). Among these are α and β thalassemias, ovalocytosis, glucose-6-phosphate dehydrogenase deficiency, and hemoglobinopathies produced by the presence of hemoglobin (Hb) S, Hb C, and Hb E. Advances have been made in our understanding by which these RBC genetic defects partially protect the bearer from the effects of the malaria infection.

Hereditary spherocytosis (HS) and hereditary elliptocytosis (HE) belong to a different category because these genetic defects have not been reported at polymorphic frequencies in any region of the world. Nevertheless, since the underlying molecular defect of these RBCs is, in many cases, a definable cytoskeletal protein abnormality, it is of great interest to investigate the fate of *P. falciparum* malaria parasites in these RBCs and to explore the possibility that host membrane proteins are necessary for intraerythrocytic parasite development beyond the initial encounter during invasion. In this paper we examine the malarial growth patterns in these abnormal RBCs. We have utilized unrelated families with spectrin-deficient HS RBCs [HS(Sp^+); nomenclature of skeletal proteins as proposed in ref. 2], although exhibiting different degrees of spectrin deficiency; HE RBCs with either complete deficiency of protein 4.1 [HE(4.1^0)] or containing a spectrin αI domain structural variant [HE($Sp\alpha^{I/65}$)]; and RBCs containing band 3 structural variants.

MATERIALS AND METHODS

Subject Material. HS and HE subjects were referred because of either acute anemia that occurred during the course of a mild febrile illness or chronic anemia. All HS subjects (AR, MR, MB, FB, LM, MP, and DM) had palpable spleen tips, except for AR, who had been splenectomized. All HS subjects and HE subjects AL, Sr., AL, Jr., and DW had elevated reticulocyte counts. HS was confirmed by RBC osmotic fragility tests, detection of increased abnormally dense RBCs (3), and spherocytes on blood smears. HE was confirmed by observation of elliptocytes on blood smears. The diagnosis of band 3 variant RBCs was described previously (4, 5).

Culture of Malaria Parasites. Clone A-2 of the FCR-3 strain (6) of *P. falciparum* was cultured in flasks (75 cm^2; Corning) (7). Cultures were maintained at 37°C in candle jars with type A^+ RBCs and plasma. Infected RBCs were maintained at 5% (vol/vol) cell suspension in complete medium [RPMI-1640 (Sigma) buffered with Tes and supplemented with glucose (2 mg/ml), gentamicin (40 μg/ml), 5-fluorocytosine (50 μg/ml), and 10% (vol/vol) human plasma (8)].

***P. falciparum* Invasion and Growth.** Normal and pathologic (HS, HE, and band 3 variant) RBCs were cultured with *P. falciparum* within 4 days of being drawn. The inoculum was the product of plasmagel treatment and had a parasitemia of 50–70% (9). Immediately after dilution with RBCs to be tested, the parasitemia was between 0.5% and 1.5%. Aliquots (1.5 ml) of parasitized RBCs (5% suspension) were added in triplicate to flat-bottom 24-well microtiter plates. For each experiment, Giemsa-stained slides were prepared daily when fresh medium was added. Parasite count involved counting 1000 RBCs per slide. Parasite invasion was measured by the uptake of [^{3}H]hypoxanthine in normal and abnormal-infected RBCs 18 hr after addition of infected RBCs (10). Levels of ATP (11) and reduced glutathione (12) present in HS RBCs

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Abbreviations: RBC, erythrocyte; HS, hereditary spherocytosis; HE, hereditary elliptocytosis.

¶To whom reprint requests should be addressed.

incubated at 37°C under culture conditions were determined. Extent of RBC hemolysis was determined by Hb content of culture supernatants (13), after the removal of any remaining RBCs by centrifugation.

Preparation of RBC Membranes. RBC ghost membranes were prepared by hypotonic lysis (14). Protease inhibitors (0.4 mM diisopropyl fluorophosphate and 0.5 mM EGTA) were present throughout the ghost preparations.

RBC Membrane Protein Determinations. RBC ghost membranes were solubilized and subjected to SDS/PAGE under reducing conditions (15). The gels were stained with Coomassie brilliant blue and the separated protein bands were quantitated by laser densitometry (Biomed).

Spectrin Tetramer/Dimer Associations. Spectrin tetramer/dimer quantitations were performed on spectrin extracts isolated from HE RBC membranes by nondenaturing gel electrophoresis (16). After staining, the gels were scanned by laser densitometry, as above, and the content of spectrin tetramers (M_r 920,000) and dimers (M_r 460,000) was quantitated.

RESULTS

HS: Parasite Growth. Invasion and growth of *P. falciparum* in RBCs from seven HS subjects were studied (Fig. 1). RBCs from HS subjects exhibited a nonsynchronous growth curve similar to normal RBCs during the initial 2–3 days of growth, but starting on day 3, for six of seven HS subjects, the parasite count continuously decreased through day 6, when the cultures were stopped (Fig. 1 *A–D*). In RBCs from one HS subject (DM), the parasite growth was indistinguishable from that in normal RBCs (Fig. 1*E*). Parasite invasion, measured by incorporation of [^{3}H]hypoxanthine, was normal for all HS subjects. When parasitized HS RBCs that exhibited decreased growth on culture day 4 were used to inoculate normal RBCs, normal growth followed (Fig. 2).

To test whether HS RBCs were intrinsically unstable in culture, RBCs from two HS subjects were preincubated at 37°C for 3 days prior to addition of parasitized RBCs. These preincubated HS RBCs sustained *P. falciparum* growth identically to the same HS RBCs that had not been preincubated prior to inoculation with infected RBCs (Fig. 3), suggesting that growth abnormalities observed in HS RBCs were not an artifact of HS RBC instability in culture. ATP and reduced glutathione levels in cultured HS RBCs were simliar to those in cultured normal RBCs. RBC hemolysis was <1% in infected cultures for both normal and HS RBCs.

HS: RBC Spectrin Content. To demonstrate sensitivity of the method used to quantitate spectrin, serial dilutions of normal RBC ghost membrane proteins were subjected to

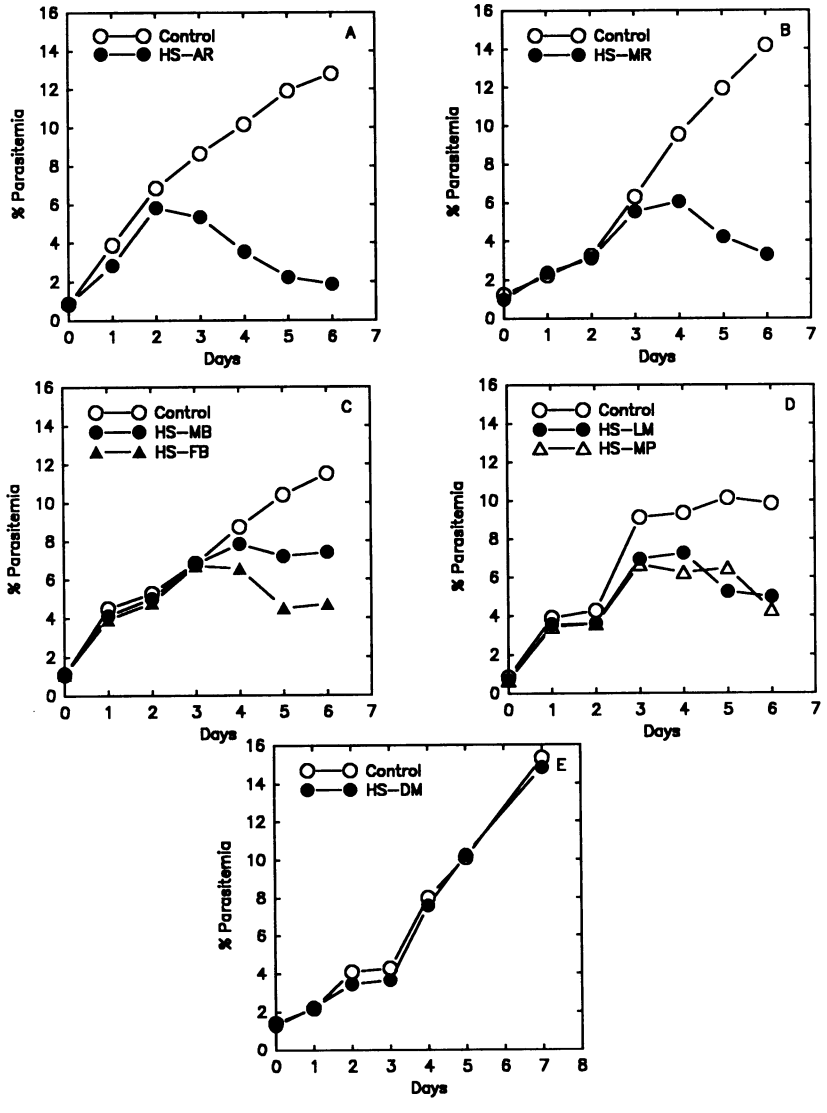

FIG. 1. Growth of *P. falciparum* in normal and HS RBCs in culture. (*A*) ○, Control; ●, HS subject AR. (*B*) ○, Control; ●, HS subject MR. (*C*) ○, Control; ●, HS subject MB; ▲, HS subject FB. (*D*) ○, Control; ●, HS subject LM; △, HS subject MP. (*E*) ○, Control; ●, HS subject DM. All parasitemia values represent the mean of three separate determinations that agreed within 0.5%.

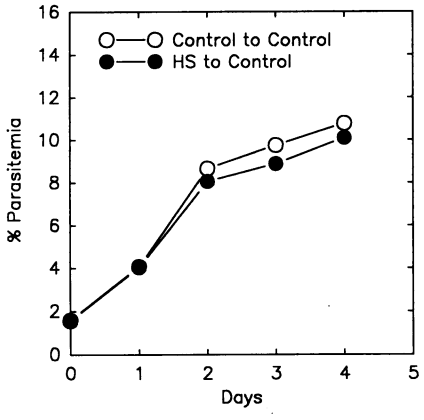

FIG. 2. Growth of *P. falciparum* in normal RBCs after prior growth in normal (○) or HS (●) RBCs. Parasitemia values represent the mean of three separate determinations that agreed within 0.1%.

SDS/PAGE and quantitative laser densitometry. Dilution of the amount of spectrin applied to the gels from 100% to 50% was paralleled by a decreased quantity of spectrin recorded by densitometry ($r = 0.99$; data not shown).

This method was used to evaluate RBC spectrin content in seven HS subjects and in normal controls (Table 1). Six of the seven HS subjects had decreased spectrin contents [HS(Sp^+)] that ranged from 12% to 33% of normal controls. One HS subject (DM) had normal spectrin levels. Decreased parasite growth in HS(Sp^+) RBCs varied directly with the extent of spectrin-deficiency in these RBCs (Fig. 4, $r = 0.899$). HS(Sp^+) RBCs were further subdivided into homogeneous cell populations by density gradient centrifugation (3). The bottom fraction of these preparations, which contained the most-dense RBCs, was then tested for parasite growth. We found that parasite growth in these dense RBCs was decreased to the same extent as growth in the unseparated (whole blood) HS(Sp^+) RBCs (Fig. 5).

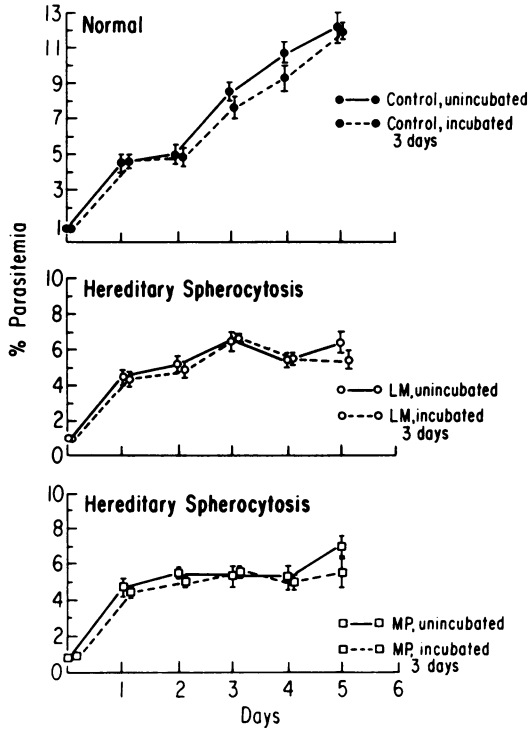

FIG. 3. Growth of *P. falciparum* in normal and HS RBCs in culture with or without preincubation of the RBCs for 3 days in RPMI-1640 complete medium under culture conditions. Results for two HS subjects (LM and MP) are shown. Each point represents the mean ± SD of three separate determinations.

Table 1. Spectrin content of HS RBCs

Source of RBCs	Spectrin content	% of control
Control	1.13 ± 0.04	100
HS subjects		
AR	0.72 ± 0.12*	67
MR	0.98 ± 0.07*	83
MB	0.97 ± 0.07*	88
FB	0.85 ± 0.11*	67
LM	0.96 ± 0.05*	85
MP	0.95 ± 0.06*	84
DM	1.14 ± 0.05	100

RBC membrane spectrin was measured by SDS/PAGE and densitometry. Results are expressed as the ratio of spectrin to band 3 and are given as the mean ± SD from three separate determinations.
*$P < 0.001$ vs. control.

HE: Parasite Growth. Invasion and growth of *P. falciparum* in RBCs from four HE subjects was studied. Three HE subjects were from one family (AL, Sr., AL, Jr., and ML). Structural studies on spectrin from these HE RBCs indicated a defect in the spectrin αI domain that gives rise to an $\alpha^{I/65}$ variant [HE($Sp\alpha^{I/65}$)] (17). Analysis of spectrin oligomeric state revealed that AL, Sr., had 27% spectrin dimers; AL, Jr., had 20% dimers; and ML had 16% dimers, in contrast to the 5–10% dimer content of spectrin in normal RBCs. The fourth HE subject (DW) had normal RBC spectrin but was completely deficient in protein 4.1 [HE(4.1^0)]. The characterization of these RBCs has been described (18).

Growth of *P. falciparum* in all of the HE RBCs was abnormal (Fig. 6). Growth abnormalities became apparent after 3 days for AL, Sr., and AL, Jr.; after 5 days for ML; and after 2 days for DW. Parasite invasion, measured by the initial incorporation of [^{3}H]hypoxanthine, was normal in all HE RBCs. Decreased parasite growth in HE RBCs was closely correlated with spectrin dimer content ($r = 0.94$; data not shown).

Band 3 Structural Variant. *P. falciparum* growth in RBCs from two unrelated subjects whose RBC membranes contained structural variants of band 3 (4, 5) was studied and found to be normal (data not shown).

DISCUSSION

The experiments reported here demonstrate that *P. falciparum* grows abnormally in RBCs with genetically defective cytoskeletal proteins. The pathologic RBC types examined—

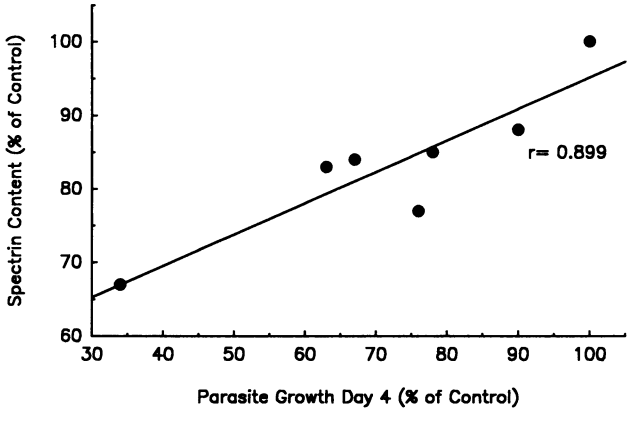

FIG. 4. Correlation of malarial parasite growth on day 4 in HS RBCs with RBC spectrin content. The line drawn represents the best fit by regression analysis, and the correlation coefficient (r) is given.

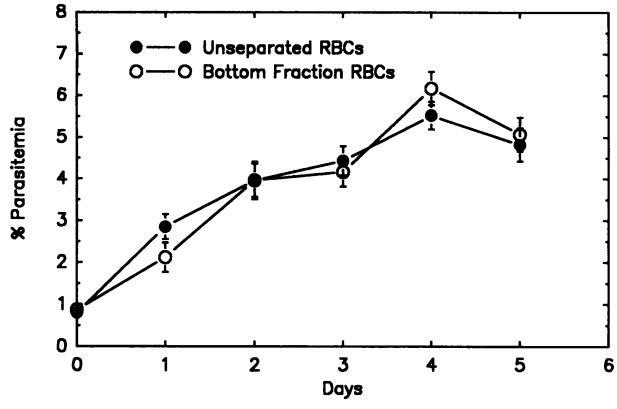

FIG. 5. Growth of *P. falciparum* in density-fractionated HS(Sp$^+$) RBCs. RBCs were fractionated on isopycnic Stractan/Percoll density gradients. The bottom fraction (●), containing the most-dense RBCs, was taken for the growth experiments and compared to growth in HS whole blood (○). Parasitemia values represent the mean ± SD of three separate determinations.

spectrin-deficient HS [HS(Sp$^+$)], HE due to protein 4.1 deficiency [HE(4.1^0)], and HE due to the presence of a spectrin αI domain structural variant [HE(Spα$^{I/65}$)]—could not sustain normal parasite growth. In contrast, parasite invasion of these RBCs was normal. Furthermore, HS without spectrin deficiency, and band 3 variants, sustained normal parasite growth.

The following questions can be asked. Are parasite growth abnormalities derived from properties of the abnormal RBCs

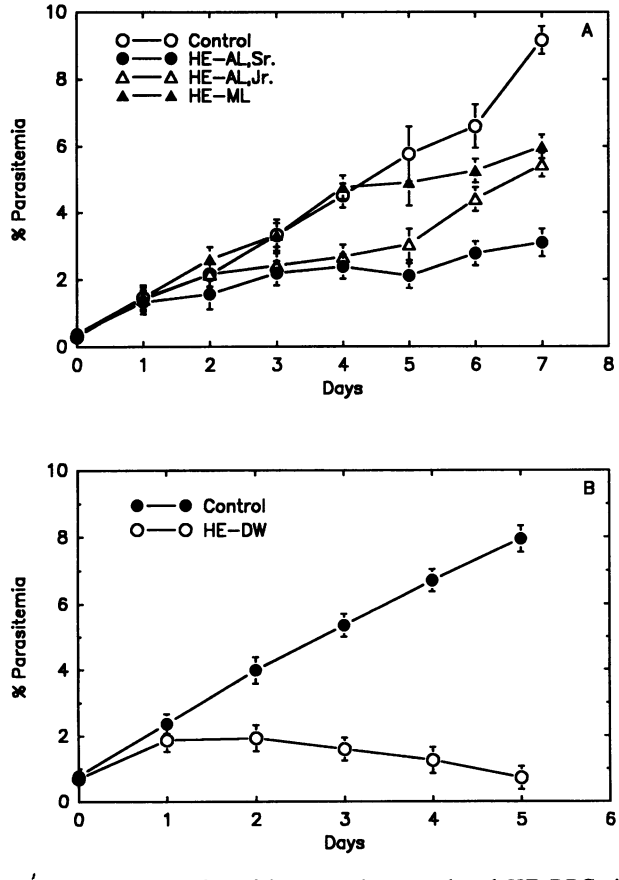

FIG. 6. Growth of *P. falciparum* in normal and HE RBCs in culture. (*A*) HE RBCs containing spectrin αI domain variants [HE(Spα$^{I/65}$)]: ○, control; ●, HE subject AL, Sr.; △, HE subject AL, Jr.; ▲, HE subject ML. (*B*) HE RBCs completely deficient in protein 4.1 [HE(4.1^0)]: ●, control; ○, HE subject DW. Parasitemia values represent the mean ± SD of three separate determinations.

(i.e., decreased deformability, abnormalities induced by culture conditions, potential secondary metabolic disruption, etc.)? Alternatively, does the parasite require a normal host cytoskeleton for its development?

The first of these alternatives seems unlikely for the following reasons. (*i*) Although HS(Sp$^+$), HE(4.1^0), and HE(Spα$^{I/65}$) RBCs lack the deformability of normal RBCs because of their cytoskeletal defects, the invasion of these RBCs by *P. falciparum* (which requires a series of deformation events) is normal. (*ii*) The possibility that the culture conditions were responsible for the parasite growth abnormalities in HS(Sp$^+$) RBCs is unlikely, since preincubation of HS(Sp$^+$) RBCs in culture for 3 days had no effect on the ability of these RBCs to sustain normal parasite growth. (*iii*) The differences in parasite growth were not due to increased hemolysis of the HS(Sp$^+$) RBCs, since no increase in Hb in the culture medium was detected. (*iv*) In terms of secondary metabolic abnormalities affecting parasite growth, four lines of evidence make this possibility highly unlikely. (*a*) RBCs from HS subject DM that contained normal amounts of spectrin sustained normal parasite growth, despite marked spherocytosis. This result makes it unlikely that features accompanying the presence of HS(Sp$^+$), such as decreased membrane deformability, spherocytic shape, and alterations in cation content, are responsible for abnormal parasite growth in HS(Sp$^+$) RBCs. (*b*) During culture, the ATP and glutathione levels of normal and HS(Sp$^+$) RBCs were indistinguishable. (*c*) Decreased potassium and increased sodium, characteristic of HS RBCs, have no effect on *P. falciparum* growth (19, 20). (*d*) A subpopulation of very dense HS(Sp$^+$) RBCs (the most dehydrated and probably the most metabolically affected RBCs) sustained parasite growth no worse than HS(Sp$^+$) whole blood.

We conclude from this analysis that the most likely explanation for the growth abnormalities in *P. falciparum*-infected HS(Sp$^+$) RBCs is that normal host cytoskeletal proteins are important for the normal development of the parasite during its erythrocytic stage. In accordance with these conclusions, we found that abnormal parasite growth varied proportionally with the extent of spectrin deficiency in HS(Sp$^+$) RBCs (r = 0.90). The growth abnormality is unique, in that it is characterized by normal growth (first 2–3 days) in all cases, followed by growth inhibition. Since parasites can grow normally in HS(Sp$^+$) RBCs for one or two cycles before abnormal development ensues, this suggests that the growth abnormality must be cumulative.

Koeweiden *et al.* (21), in an earlier study of the growth of *P. falciparum* in normal and HS RBCs, found no difference in parasite growth rates for HS compared to normal RBCs. Possible explanations for the disparity with our findings may be (*i*) that those authors followed parasite growth for only 3 days, which is probably too short a time for significant growth abnormalities to become apparent, and (*ii*) that the HS RBCs used by those authors may have had normal amounts of spectrin, as did HS subject DM in our study.

Parasite growth in HE(Spα$^{I/65}$) RBCs, exhibiting increased content of spectrin dimers, also started to differ from normal RBCs after entering the second growth cycle (day 3). The extent of decreased growth was found to correlate with spectrin dimer content (r = 0.94). *P. falciparum* growth in protein 4.1-deficient RBCs [HE(4.1^0)] was normal only on day 1, demonstrating that the parasites could invade these RBCs but could not undergo further normal development.

Finally, qualitative differences in the band 3 structural variant RBCs did not affect parasite growth. This result is not surprising, since it has been reported that band 3 is proteolyzed by the parasite during its growth phase (22) and might not be a membrane protein essential for parasite development.

There are several potential mechanisms whereby cytoskeletal protein defects may disrupt the maturation of *P. falciparum*. For instance, the parasite may utilize host cytoskeletal proteins to construct its own membranes. Alternatively, host membrane proteins may interact with parasite proteins during intraerythrocytic growth. In either possibility, abnormal host membrane cytoskeletal proteins could impair normal parasite development.

Concerning the first mechanism, developing parasites are known to construct a parasitophorous vacuole membrane (PVM), which covers the developing ex-merozoite. Morphologic evidence suggests that this membrane is derived from the host RBC membrane (23). Abnormal host membrane proteins, such as spectrin or protein 4.1, could, therefore, become incorporated into the PVM. However, immunoelectron microscopy studies using antibodies to cytoskeletal proteins did not detect spectrin in the PVM (24, 25), although one study (25) identified spectrin clusters around the "knobs" of infected-cell membranes. Atkinson *et al.* (24) identified ankyrin, a membrane protein closely associated with spectrin (26, 27), with the flat Maurer's clefts. This finding is of particular interest, since recent genetic evidence suggests that ankyrin deficiency may be the primary cytoskeletal protein defect in some cases of HS, and that spectrin deficiency occurs as a consequence of the ankyrin abnormality (28).

To begin the examination of these hypotheses, we used *P. falciparum*-infected HS(Sp^+) RBCs (subject AR) on day 4 of culture as an inoculum to study the growth characteristics in normal RBCs of merozoites released from HS(Sp^+) RBCs. We found normal parasite invasion and growth in normal RBCs of these HS(Sp^+) "conditioned" merozoites. This result suggests that the invasion-related proteins on the surface of merozoites grown in HS(Sp^+) RBCs are normal. These results also imply that merozoites grown in HS(Sp^+) RBCs are intrinsically capable of normal development, so long as they are not reexposed to HS(Sp^+) RBCs. Taken together, these results point to extramerozoite causes for growth abnormalities in HS(Sp^+) RBCs. Experimental tests of this will require the development of reliable methods to prepare pure parasite membranes without host membrane contamination.

The second potential mechanism for decreased parasite growth stems from findings establishing that the host RBC membrane interacts, during the growth phase, with antigens synthesized by the parasite. Examples include the knob-associated histidine-rich-protein (KAHRP), which is required for knob formation (22) and endothelium adhesion (29); mature-parasite-infected-erythrocyte surface antigen (MESA), which binds to protein 4.1 in the host membrane (30); and the ring-infected-erythrocyte surface antigen (RESA), which binds (during early parasite development) to the inner surface of the host membrane, where it becomes phosphorylated (31). Taken together, these findings raise the possibility that abnormal host RBC cytoskeletal proteins could fail as a receptor, or target, for interactions with these parasite proteins, interfering with yet unrecognized parasite developmental processes. Further work will be needed to understand the complex relationships between abnormal parasite growth and host membrane proteins.

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