



Title	Antibodies reactive with the N-terminal domain of Plasmodium falciparum serine repeat antigen inhibit cell proliferation by agglutinating merozoites and schizonts
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## Antibodies Reactive with the N-Terminal Domain of *Plasmodium falciparum* Serine Repeat Antigen Inhibit Cell Proliferation by Agglutinating Merozoites and Schizonts

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**The serine repeat antigen (SERA) is a vaccine candidate antigen of *Plasmodium falciparum*. Immunization of mice with *Escherichia coli*-produced recombinant protein of the SERA N-terminal domain (SE47') induced an antiserum that was inhibitory to parasite growth in vitro. Affinity-purified mouse antibodies specific to the recombinant protein inhibited parasite growth between the schizont and ring stages but not between the ring and schizont stages. When Percoll-purified schizonts were cultured with the affinity-purified SE47'-specific antibodies, schizonts and merozoites were agglutinated. Indirect-immunofluorescence assays with unfixed parasite cells showed that SE47'-specific immunoglobulin G (IgG) bound to SERA molecules on rupturing schizonts and merozoites but the IgG did not react with the schizont-infected erythrocytes (RBC). Furthermore, double-fluorescence staining against SE47'-specific IgG and anti-human RBC membrane IgG showed that the RBC membrane disappeared from SE47'-specific-IgG-bound schizonts after cultivation. These observations suggest that the SE47'-specific antibodies inhibit parasite growth by cross-linking SERA molecules that are associated with merozoites in rupturing schizonts with partly broken RBC and parasitophorous vacuole membranes, blocking merozoite release.**

Malaria remains one of the most devastating human infectious diseases. The appearance of malaria parasites with resistance to antimalarial drugs and of mosquito vectors with resistance to insecticides has made it more difficult to cure and prevent malaria infection, respectively. It is therefore of increasing importance to develop malaria vaccines.

The *Plasmodium falciparum* serine repeat antigen (SERA) is one of the malaria vaccine candidate antigens against the asexual blood stage (for reviews, see references 1 and 18). SERA, also known as SERP (17) and pl26 (6, 7) is an asexual blood stage antigen produced in large amounts specifically during late trophozoite and schizont stages (2, 5). SERA was originally identified by a mouse monoclonal antibody (immunoglobulin M [IgM]) that inhibits parasite growth in vitro (2, 13). Immunization of Aotus and squirrel monkeys with a recombinant protein comprising part or all of the N-terminal domain (47 kDa) of SERA conferred significant protection from challenge infection (14–16, 27).

SERA protein (from the Honduras-1 strain of *P. falciparum*) contains 989 amino acids, including a repetition of 35 serine residues (5), and has limited sequence homology with the active site of serine proteases (9, 12). However, the physiological function of SERA is poorly characterized. Comparison of the sequences of several allelic forms of SERA showed limited diversity in the N-terminal region, and the majority of the diversity is due to deletion or insertion events rather than point mutations (22). The recent analysis of the *P. falciparum* chromosome 2 genome sequence revealed that the SERA gene and seven SERA gene homologues are clustered (11).

The SERA polypeptide is secreted primarily into the lumen of the parasitophorous vacuole after removal of its 22-amino-

acid signal peptide (6). Coincident with the release of merozoites, a large fraction of the total pool of the 126-kDa SERA protein is proteolytically processed into a 47-kDa N-terminal domain, a 50-kDa central domain, an 18-kDa C-terminal domain, and a poorly characterized 6-kDa domain (8). There are conflicting reports arguing the presence or absence of SERA on the merozoite surface (2, 25). SERA has recently been identified as a phospholipid-binding protein that can recognize inner-leaflet phospholipids of the host erythrocytes (RBC) (25).

Vaccination of rodents or goats with recombinant SERA N-terminal-domain protein elicits antibodies that inhibit the growth of the parasite in vitro (3, 4, 10, 24, 26). It is therefore of great interest to elucidate the mechanism of inhibition of the parasite's proliferation by anti-SERA antibodies. In this report, we examine the effect of the affinity-purified SE47'-specific IgG on the parasite's development in RBC. The results demonstrate that the SE47'-specific IgG inhibited parasite growth by a mechanism involving agglutination of merozoites and rupturing of schizonts.

### MATERIALS AND METHODS

**Protein expression and purification.** The recombinant SE47' protein (amino acids 17 to 382) was expressed in XL1-Blue cells by using a synthetic SERA gene and was purified according to the method of Sugiyama et al. (26) and Barr et al. (3). Briefly, 10 g of *Escherichia coli* cells containing induced SE47' protein was suspended in STE buffer (50 mM Tris-HCl [pH 8.0]–5 mM EDTA–25% sucrose–5 mM 2-mercaptoethanol) with 0.1 mg of lysozyme ml<sup>-1</sup> and sonicated. After centrifugation, ammonium sulfate was added to the supernatant at a final 30% saturation to precipitate the SE47' protein. The precipitate was collected by centrifugation and dissolved in TEGB buffer (10 mM Tris-HCl [pH 7.6]–1 mM EDTA–10% glycerol–10 mM 2-mercaptoethanol) containing 8 M urea and 0.1% sodium dodecyl sulfate (SDS). The solution was dialyzed against phosphate-buffered saline (PBS) (10 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 100 mM NaCl, pH 6.8) containing 0.1% SDS and was then subjected to gel filtration on TSK gel G 4000 SW (Tosoh, Tokyo, Japan). The chromatography was carried out at a flow rate of 1 ml/min, and 0.5-ml fractions were collected. Fractions 34 and 35, which contained SE47' protein, were pooled and mixed with 100 mM 2-mercaptoethanol. The pooled fraction was then heated at 80°C for 15 min and subjected to the

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same column chromatography. The SE47' protein that was recovered in fractions 39 and 40 was concentrated by a membrane filter unit, Centrprep 10 (Amicon). The preparations yielded 15 mg of SE47' protein. The protein was dialyzed against PBS (1.9 mM  $\text{NaH}_2\text{PO}_4$ , 8.1 mM  $\text{Na}_2\text{HPO}_4$ , 154 mM NaCl, pH 7.2) containing 0.1% SDS and kept at  $-20^\circ\text{C}$ .

**Immunization of mice.** Mice (6-week-old female BALB/c; Japan SLC Inc.) were immunized with SE47' and Freund's adjuvant (Difco) by subcutaneous injection on days 0, 14, and 28. Each mouse received 50  $\mu\text{g}$  of the protein at the initial injection followed by 25  $\mu\text{g}$  of the protein at the second and third injections. The SE47' protein was emulsified at a 1:1 ratio with Freund's complete adjuvant for the initial injection and with Freund's incomplete adjuvant for the second and third injections. For control serum, five mice were immunized with PBS containing 0.1% SDS by the procedures described above. On day 35, blood was collected in fresh Eppendorf tubes and incubated at  $37^\circ\text{C}$  for 1 h, followed by 12 h at  $4^\circ\text{C}$ . The sera were then separated by centrifugation at  $1,250 \times g$  for 10 min. All sera were heat inactivated at  $56^\circ\text{C}$  for 30 min and stored in aliquots at  $-20^\circ\text{C}$ .

**ELISA and Western blot analyses.** Enzyme-linked immunosorbent assay (ELISA) was performed as described previously (24, 26). One microgram of SE47' protein was used to coat each well of a 96-well MaxiSorp dish (Nunc). SE47'-specific IgG in a serum was detected with biotinylated horse IgG specific to mouse IgG (Vector Laboratories) as a secondary antibody. Avidin-conjugated peroxidase (ABC kit; Vector Laboratories) and 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) were used for ELISA. The ELISA titer was determined by a cutoff absorbance of 0.2 at 405 nm with a microtiter plate reader (Titertek Multiskan MCC/340 MKII). The purified recombinant SE47' protein and a cell homogenate of *P. falciparum* (FCR3) (24) were used for the Western blot analyses (26). Affinity-purified SE47'-specific IgG and biotinylated horse IgG specific to mouse IgG were used as primary and secondary antibodies, respectively. Avidin-conjugated peroxidase and diaminobenzidine tetrahydrochloride were used for the Western blot analysis.

**Preparation of IgG.** Total IgG from mouse serum was prepared on a Hi-Trap protein A-Sepharose column (Pharmacia). Pooled anti-SE47' serum (10 ml) or pooled control serum (2.0 ml) was diluted 10-fold with PBS (1.4 mM  $\text{KH}_2\text{PO}_4$ , 4.3 mM  $\text{Na}_2\text{HPO}_4$ , 2.7 mM KCl, 137 mM NaCl, pH 8.0) and applied to the column (bed volume, 5 and 1 ml, respectively). Total IgG was eluted from the column with 0.1 M citrate buffer (pH 2.5). The concentration of IgG was determined by measuring optical density at 280 nm (an absorbance of 1.4 is equivalent to 1.0 mg of IgG  $\text{ml}^{-1}$ ). SE47'-specific IgG was prepared from total IgG by affinity chromatography with Sepharose 4B (Pharmacia) covalently linked with SE47' protein. The bound IgG was eluted with 0.1 M glycine-HCl (pH 2.5) and was immediately neutralized with 1/10 volume of 1 M Tris-HCl (pH 8). The eluted fractions were extensively dialyzed against PBS (1.4 mM  $\text{KH}_2\text{PO}_4$ , 4.3 mM  $\text{Na}_2\text{HPO}_4$ , 2.7 mM KCl, 137 mM NaCl, pH 8.0) and then dialyzed against a basal medium (RPMI 1640) for the parasite culture. The SE47'-specific IgG was concentrated by Centrprep 10 to 3 mg  $\text{ml}^{-1}$  and stored at  $-20^\circ\text{C}$ . In a typical preparation, 1.5 mg of SE47'-specific IgG was obtained from 4 mg of total IgG that was prepared from 1 ml of antiserum. Nonspecific IgG was prepared from control mouse serum with a Hi-Trap protein A-Sepharose column (Pharmacia) as described above. When the nonspecific IgG was applied to this SE47' affinity column, the adsorbed IgG was undetectable.

**Parasite cell preparation.** *P. falciparum* FCR3 was maintained in culture according to the methods of Trager and Jensen (29) and Sugiyama et al. (26). The parasites were grown in a 5%  $\text{O}_2$  and 5%  $\text{CO}_2$  atmosphere with 2% (vol/vol) type O RBC in a culture medium containing 10% heat-inactivated human type O serum. Synchronization of parasite growth was done by two consecutive D-sorbitol treatments (5%) with a 30-h interval (30). This method produced early ring stage parasites with a 2- to 6-h window of age span after invasion. RBC infected by late trophozoites and schizonts were isolated by 63% (vol/vol) Percoll (Pharmacia) density centrifugation from a synchronized culture as described by Tosta et al. (28).

Free merozoites were isolated as follows. Percoll-purified RBC infected by trophozoites and schizonts were cultured in complete medium without fresh RBC. After 3 h of cultivation, the culture was centrifuged at  $200 \times g$  for 3 min. The supernatant was filtered through 2- $\mu\text{m}$ -pore-size membrane sieves (prefilter) (Millipore) according to the method of Mrema et al. (23) and was immediately used for further experiments. The free merozoites were visualized by Giemsa staining.

**Growth inhibition and invasion assay.** Parasite growth inhibition assays were performed in a medium containing 2% RBC with a 0.5% initial parasitemia in the presence of the indicated mouse IgG or serum for 24 or 72 h in a 96-well microtiter plate. The culture medium was replaced every 24 h with fresh medium with or without the indicated IgG. Parasite growth was examined by Giemsa staining of thin smears, and the parasitemia was scored by counting over 5,000 RBC in a slide. The observed parasitemia was divided by the parasitemia of the indicated control culture to give the percent growth inhibition.

**Assay for parasite cell agglutination.** Percoll-purified RBC infected by trophozoites and schizonts were cultured in microculture plates (100  $\mu\text{l}$  per well) in the presence of the indicated IgG at a density of  $10^5$  schizonts  $\text{ml}^{-1}$  without fresh RBC. After cultivation for the indicated time, all of the culture was smeared on a glass slide and Giemsa stained. Mature schizonts, agglutinated merozoites, and single merozoites were counted over all areas of the smear.

Purified merozoites ( $10^5$ ) were incubated in 100  $\mu\text{l}$  of complete medium containing 100  $\mu\text{g}$  of the indicated mouse IgG  $\text{ml}^{-1}$  without fresh RBC at  $37^\circ\text{C}$  for 30 min. The agglutinated merozoites and free merozoites were counted in Giemsa-stained thin smears as described above.

**Immunofluorescence assay.** Percoll-purified RBC infected by trophozoites and schizonts were cultured for 0, 4, or 8 h at a density of  $10^6$   $\text{ml}^{-1}$  in a complete medium containing 100  $\mu\text{g}$  of the indicated IgG  $\text{ml}^{-1}$  at  $37^\circ\text{C}$ . After cultivation, the parasite cells were washed three times by centrifugation at  $1,000 \times g$  for 3 min and were subjected to immunofluorescence assays. The cells were resuspended in 100  $\mu\text{l}$  of PBS-3% bovine serum albumin (BSA) containing fluorescein isothiocyanate (FITC)-conjugated goat IgG against mouse IgG (ICN Pharmaceuticals, Inc.-Cappel Products) diluted 1:1,000 and 1  $\mu\text{g}$  of diamidinophenylindole (DAPI) (catalog no. D-1388; Sigma)  $\text{ml}^{-1}$ . After incubation for 30 min at  $37^\circ\text{C}$ , the parasite cells were washed five times with PBS and fixed in PBS containing 3% paraformaldehyde. The fixed cells were mounted with PermaFluor aqueous mounting medium (Immunon) and inspected by fluorescence microscopy (Axioskop; Zeiss). For the double-fluorescence assay, Percoll-purified trophozoites and schizonts were cultured in a complete medium containing the indicated IgG and washed as described above. The cells were then incubated in PBS-3% BSA containing rabbit IgG against human RBC membrane (ICN Pharmaceuticals, Inc.-Cappel Products) diluted 1:5,000 at  $37^\circ\text{C}$  for 30 min. After being washed three times with PBS, the cells were resuspended in PBS-3% BSA containing FITC-conjugated goat IgG against mouse IgG, 1  $\mu\text{g}$  of DAPI  $\text{ml}^{-1}$ , and FluoroLinkCy3-labeled goat IgG against rabbit IgG (Amersham) diluted 1:1,000. The parasite cells were washed and mounted for fluorescence assay as described above.

Merozoites were fixed immediately after purification with 2% paraformaldehyde in PBS on ice for 30 min and spread on a slide. After being air dried, the slides were incubated for 30 min at  $37^\circ\text{C}$  in PBS-3% BSA and subsequently reacted with the SE47'-specific IgG in PBS-3% BSA. The slides were washed three times in PBS and then incubated with secondary antibody (FITC-conjugated goat IgG against mouse IgG) in PBS containing 1  $\mu\text{g}$  of DAPI  $\text{ml}^{-1}$  to stain the parasite nuclei. The slides were washed five times in PBS, mounted, and inspected as described above.

## RESULTS

**SE47'-specific IgG inhibits *P. falciparum* growth.** The anti-serum from mice immunized with recombinant SE47' protein was prepared as described in Materials and Methods. The ELISA titer of anti-SE47' serum was 87,000, while that of the control serum was <50. The parasite growth inhibition assay was carried out with 5% (vol/vol) anti-SE47' serum. After incubation for 72 h, the parasitemia was 2%, while the parasitemias in the media with the control serum and without serum were 7.2 and 9.1%, respectively. To verify that parasite growth is inhibited by IgG specific to SE47' protein in the prepared antiserum, we purified total and SE47'-specific IgG from mouse antiserum with protein A and Sepharose 4B covalently cross-linked with recombinant SE47' protein. The cross-reactivity of the purified SE47'-specific IgG to both the parasite SERA protein and recombinant SE47' protein was confirmed by Western blot analysis (Fig. 1). The total IgG and the SE47'-specific IgG were subjected to the parasite growth inhibition assay. Parasite growth was inhibited by both total IgG and SE47'-specific IgG in a concentration-dependent manner (Fig. 2). Maximum inhibition (80%) was obtained with the SE47'-specific IgG at 100  $\mu\text{g}$   $\text{ml}^{-1}$  after cultivation for 72 h; however, further inhibition was not observed with increasing concentrations of the IgG.

**SE47'-specific IgG inhibits *P. falciparum* development from schizont to ring.** To determine the parasite developmental stage where the primary inhibition by SE47'-specific IgG occurs, the parasite cells were synchronized. Synchronized ring or trophozoite and schizont stage parasites were grown for 24 h in the presence of SE47'-specific IgG or nonspecific IgG or without IgG. All ring stage parasites developed to trophozoites and schizonts in the presence of SE47'-specific IgG (Fig. 3A). In contrast, initially inoculated trophozoites and schizonts (0.5% parasitemia) developed to 0.54% rings and 0.21% schizonts in the presence of SE47'-specific IgG while trophozoites and schizonts developed to 2.5% parasitemia of rings and 0% parasitemia of trophozoites and schizonts in the presence of non-

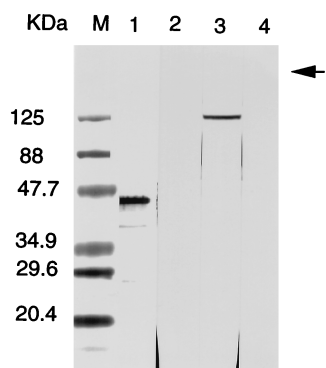


FIG. 1. Western blot of *P. falciparum* cell homogenate and recombinant SE47' protein with affinity-purified SE47'-specific IgG. The purified recombinant SE47' protein (lanes 1 and 2) and Percoll-purified trophozoites and schizonts,  $10^5$  cells of *P. falciparum* FCR3 (lanes 3 and 4), were run in 6 to 12% gradient SDS-polyacrylamide gels under nonreducing conditions. The arrow indicates the gel top. Western blotting was performed with SE47'-specific IgG (lanes 1 and 3) or nonspecific IgG (lanes 2 and 4).

specific IgG or no IgG (Fig. 3B). These results indicate that SE47'-specific IgG inhibits parasite development from the schizont stage to the ring stage but not the intracellular development of the parasite from ring stage to schizont stage.

When Percoll-purified trophozoites and schizonts were cultured with various concentrations of SE47'-specific IgG for 24 h, development to the ring stage was inhibited as a function of the antibody concentration up to  $100 \mu\text{g ml}^{-1}$  (Fig. 4). The maximum inhibition observed was 60% in one cycle of parasite cell proliferation. This observation was similar to the result shown in Fig. 2 and further substantiated the inhibitory effect of SE47'-specific IgG on parasite development from schizonts to rings.

**SE47'-specific IgG agglutinates schizonts and merozoites.** The effect of SE47'-specific IgG on parasite cell development was further analyzed by using purified RBC infected by trophozoites and schizonts. Percoll-purified parasite cells the majority of which were schizonts (90% schizonts and 10% late trophozoites) were cultured with SE47'-specific or nonspecific

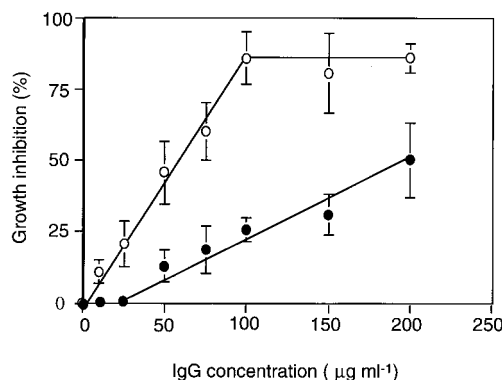


FIG. 2. *P. falciparum* parasite growth inhibition by mouse IgG specific to SE47'. Cells of *P. falciparum* FCR3 at late trophozoite and schizont stages were cultured for 72 h with or without mouse IgG. Total IgG (solid circles) and SE47'-specific IgG (open circles) were prepared from anti-SE47' mouse antisera as described in Materials and Methods. The initial parasitemia was 0.5%. The parasitemia of the control culture with nonspecific IgG ( $200 \mu\text{g ml}^{-1}$ ) or without IgG after 72 h of cultivation was 5.42 or 5.61%, respectively. Percent growth inhibition (shown as means  $\pm$  standard deviations;  $n = 4$ ) was calculated by using the parasitemia of the culture grown with nonspecific IgG.

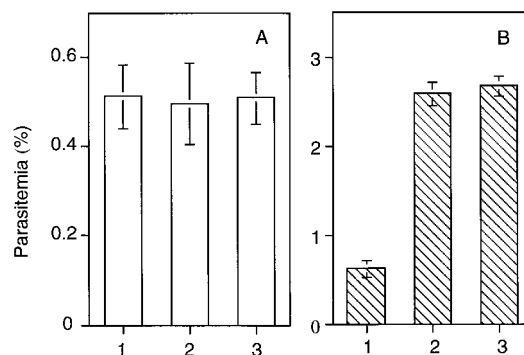


FIG. 3. Stage specificity of the inhibitory effect of the SE47'-specific IgG on *P. falciparum* growth. Synchronized *P. falciparum* FCR3 cells were cultured for 24 h in the presence of  $100 \mu\text{g}$  of SE47'-specific IgG (bars 1) or nonspecific IgG (bars 2)  $\text{ml}^{-1}$  or without IgG (bars 3). (A) Initial parasitemia was 0.5% at ring stage. (B) Initial parasitemia was 0.5% at late trophozoite and schizont stages. Both the results for intraerythrocyte growth and those for invasion are given as the mean percentage of parasitemia with a standard error (mean  $\pm$  standard deviation;  $n = 3$ ).

IgG in the absence of fresh RBC. After 4 and 8 h of incubation with SE47'-specific IgG, agglutinated schizonts and agglutinated merozoites were observed by Giemsa staining (Fig. 5B and C). In a control culture with nonspecific IgG, predominantly single rupturing schizonts and single merozoites were observed (Fig. 5F and G). To examine whether merozoites were agglutinated with SE47'-specific IgG, isolated merozoites were incubated with each IgG. The merozoites were agglutinated by SE47'-specific IgG but not by nonspecific IgG, as shown in Fig. 5D and H. The presence of SERA on merozoites was confirmed by FITC assay (see Fig. 7, row 5).

For quantitative analysis, total single and agglutinated merozoites and schizonts were counted. In a culture with nonspecific IgG, the number of single merozoites increased and that of schizonts decreased with cultivation time (Fig. 6A and C). In contrast, the number of single merozoites decreased and that of agglutinated merozoites increased in the presence of SE47'-specific IgG (Fig. 6A and B). The number of schizonts remaining after 10 h of cultivation was higher in SE47'-specific IgG than in nonspecific IgG (Fig. 6C). These results indicate that SE47'-specific IgG agglutinates both merozoites and schizonts,

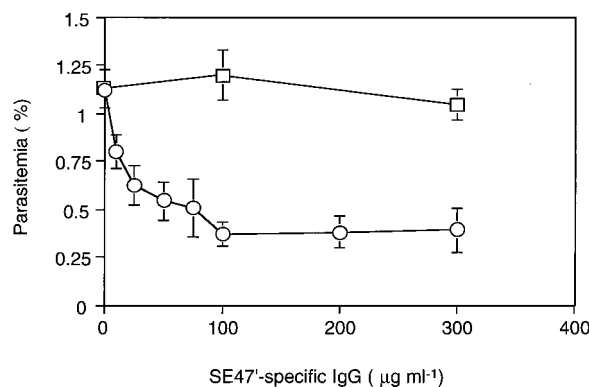


FIG. 4. *P. falciparum* parasite invasion inhibition by mouse IgG specific to SE47'. The growth inhibition assay was carried out with Percoll-purified trophozoite and schizont parasite cells in medium containing the indicated concentrations of SE47'-specific IgG (circles) or nonspecific IgG (squares). The initial parasitemia was 0.5%. The parasitemias were counted after 24 h on each culture (shown as means  $\pm$  standard deviations;  $n = 3$ ).



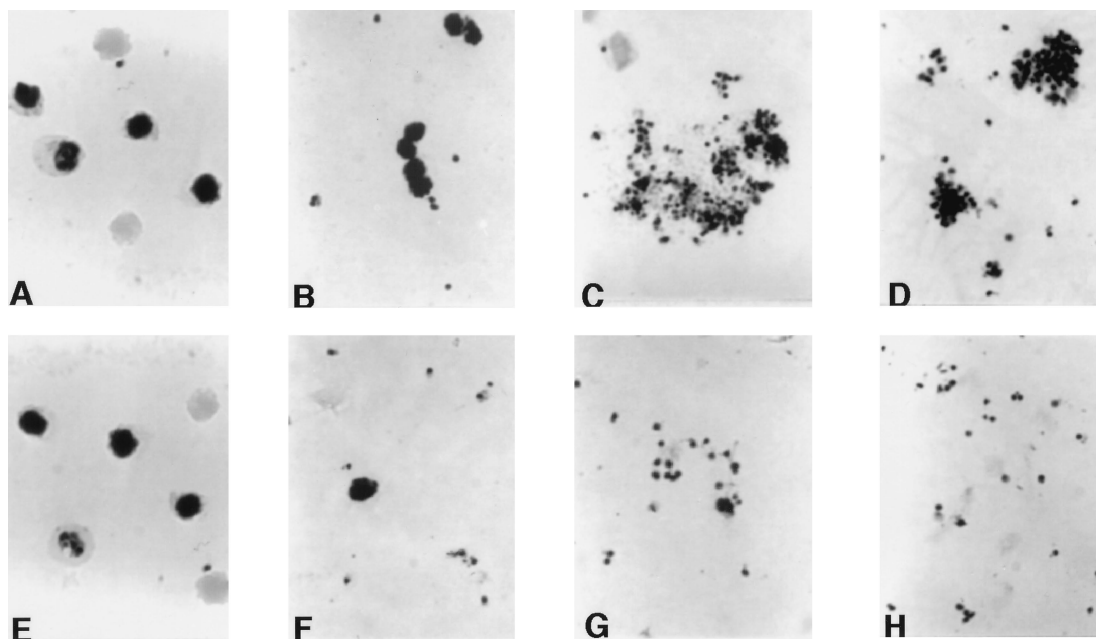


FIG. 5. SE47'-specific-IgG-mediated parasite cell agglutination. Percoll-purified trophozoites and schizonts were cultured at 37°C in a medium containing 100  $\mu$ g of SE47'-specific IgG (A, B, and C) or nonspecific IgG (E, F, and G)  $\text{ml}^{-1}$ . After incubation for 0 (A and E), 4 (B and F), and 8 (C and G) h, the parasite cells were Giemsa stained. Merozoites were isolated as described in Materials and Methods. Soon after isolation, the merozoites were incubated with 100  $\mu$ g of SE47'-specific IgG (D) or nonspecific IgG (H)  $\text{ml}^{-1}$  at 37°C for 30 min and Giemsa-stained.

causing depletion of single merozoites. The decrease of single merozoites was quantitatively correlated with parasite growth inhibition (Fig. 3).

**Localization of SE47'-specific IgG in agglutinated parasite cells.** To examine whether SE47'-specific IgG was present in the agglutinated parasite cells, Percoll-purified RBC infected by trophozoites and schizonts were cultivated with SE47'-specific IgG for 4 and 8 h and then reacted with FITC-conjugated goat IgG against mouse IgG. The FITC assay was carried out without fixation of the parasite cells to observe the distribution of SE47'-specific IgG under physiological conditions. Both agglutinated schizonts and merozoites were strongly fluorescent in a FITC assay (Fig. 7). The results showed that SE47'-specific IgG was present in the agglutinated parasite cells. When freshly prepared schizonts were subjected to FITC assay without fixation, fluorescence was not observed. Schizonts permeated by paraformaldehyde fixation, however, were fluorescent

(Fig. 7). These results were consistent with previous observations that SERA molecules are not on the infected RBC membrane but are predominantly in the parasitophorous vacuole (7, 17).

Under microscopic examination of the Giemsa-stained parasite cells, freshly prepared schizonts appeared to be surrounded by the RBC membrane; however, schizonts cultured for 4 to 8 h appeared to have no RBC membrane (data not shown). If the rupturing schizonts break RBC membranes before the release of merozoites, anti-SERA IgG can bind SERA in the parasitophorous vacuole. Double-immunofluorescence assays were performed to investigate the presence of RBC membranes surrounding the agglutinated schizonts in the presence of SE47'-specific IgG without fixation. When Percoll-purified schizonts were immediately subjected to double-immunofluorescence assay, Cy3 fluorescence from rabbit IgG against human RBC membrane was apparent. However, FITC fluorescence from SE47'-specific IgG against SERA was not observed (Fig. 8, column T = 0 h). After cultivation with SE47'-specific IgG for 4 h, a majority of schizonts were agglutinated and both Cy3 and FITC fluorescences were observed (Fig. 8, column T = 4 h). An additional 4 h of cultivation caused a disappearance of Cy3 fluorescence but did not effect FITC fluorescence (Fig. 8, column T = 8 h).

## DISCUSSION

In previous work, we found that affinity-purified SE47'-specific mouse IgG inhibits *P. falciparum* growth in in vitro culture and that the growth inhibition was enhanced by active complement through the classical pathway (24). However, it was not clear how antibodies against SERA inhibit parasite growth. In this study, we demonstrate that SE47'-specific IgG inhibits parasite growth at a stage between schizont and ring. We also showed that SE47'-specific IgG binds to the rupturing schizont, causing agglutination of the parasite cells and thus pre-

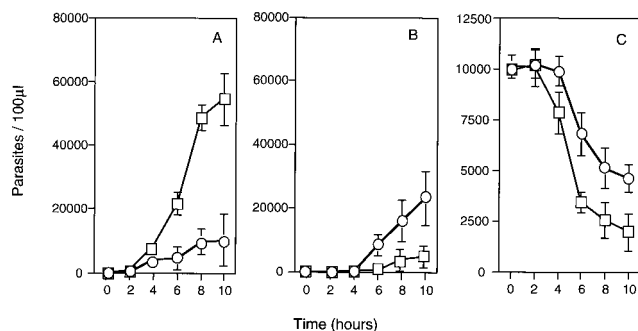


FIG. 6. Number of agglutinated parasite cells with SE47'-specific IgG. Percoll-purified trophozoites and schizonts ( $10^4$ ) were cultured with nonspecific IgG (squares) or SE47'-specific IgG (circles) for 0 to 10 h in 100  $\mu$ l of medium. The total numbers of single merozoites (A), agglutinated merozoites (B), and schizonts (C) in the cultures are shown (means  $\pm$  standard deviations;  $n = 3$ ).

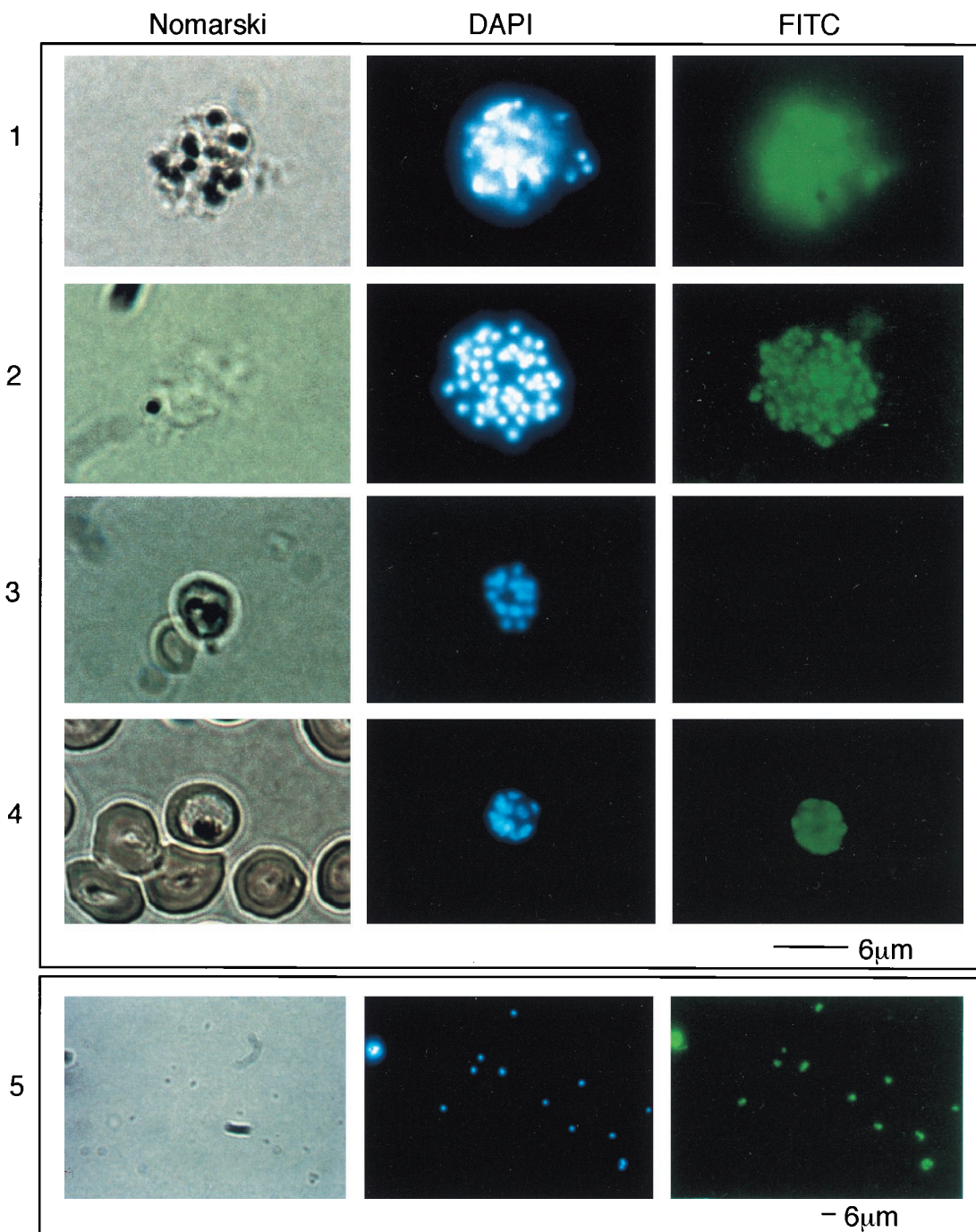


FIG. 7. Agglutinated parasite cells are surrounded by SE47'-specific IgG. Percoll-purified trophozoites and schizonts were cultured with 100 µg of SE47'-specific IgG ml<sup>-1</sup> for 4 and 8 h, and the parasite cells were washed and stained with FITC-conjugated goat anti-mouse IgG. Rows: 1, agglutinated schizonts after 4 h of cultivation; 2, agglutinated merozoites after 8 h of cultivation; 3, freshly purified single schizonts; 4, freshly purified single schizonts after paraformaldehyde fixation; 5, isolated merozoites. Nomarski, differential interference microscope image.

venting the release of free merozoites. It is highly probable that the inhibition of free-merozoite release by SE47'-specific IgG causes the parasite growth inhibition, although the antibodies may also block the binding of merozoites to RBC.

SERA molecules are not exposed to the outside of the RBC membranes of schizont stage parasites immediately after isolation with Percoll. This observation is consistent with previous reports that SERA primarily localizes in the parasitophorous

vacuole (7, 17). When probed with anti-human RBC antibodies, the RBC membrane around schizonts was dissociated during cultivation (Fig. 8). Although the process of schizont rupture is not yet clear, single merozoites would be released after breaking of both RBC and parasitophorous membranes. SE47'-specific IgG could invade the parasitophorous vacuole when both the RBC and parasitophorous membranes begin to rupture. Since SERA molecules were observed by



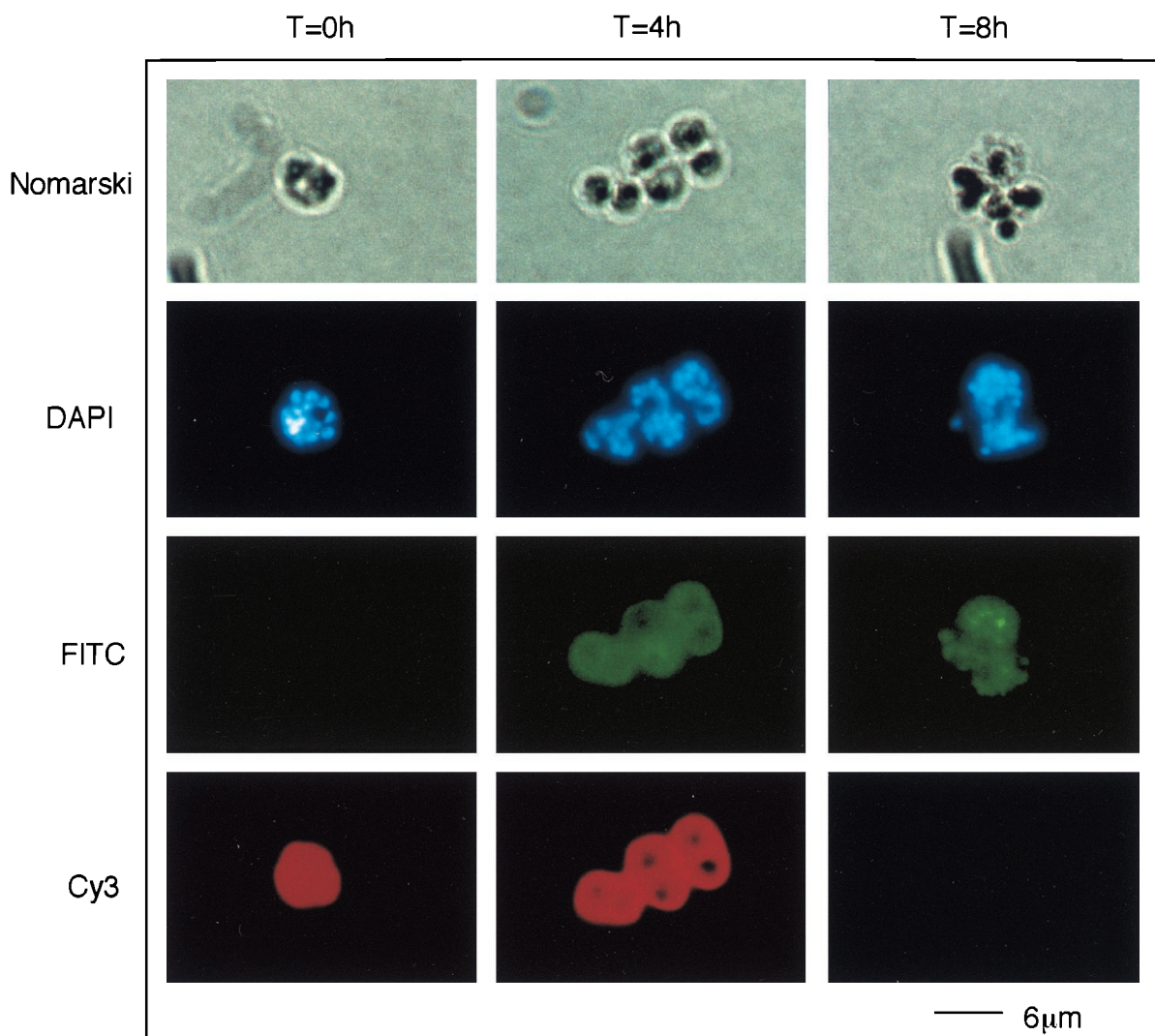


FIG. 8. Double-immunofluorescence staining of SE47'-specific IgG and RBC membranes in schizonts. Percoll-purified trophozoites and schizonts were cultured with  $100 \mu\text{g}$  of SE47'-specific IgG  $\text{ml}^{-1}$  for 0, 4, and 8 h, and the parasite cells were washed and reacted with rabbit IgG against RBC membrane. Cy3- or FITC-conjugated secondary antibodies were used to identify RBC membranes (red fluorescence) and SE47'-specific IgG (green fluorescence). Columns: T=0h, fresh schizonts before cultivation; T=4h, agglutinated schizonts after 4 h of cultivation; T=8h, agglutinated schizonts after 8 h of cultivation. Normarski, differential interference microscope image.

immuno-electronmicroscope around each merozoite in a schizont-infected RBC (23a), SE47'-specific IgG would cross-link merozoites, preventing merozoite dispersal. Further examination of rupturing schizonts is necessary to understand the status of the host RBC membrane and the parasite vacuole membrane(s). Our proposed model for parasite growth inhibition is shown in Fig. 9.

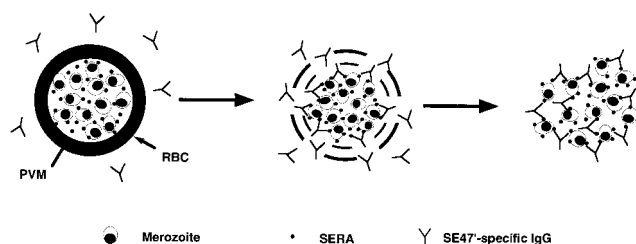


FIG. 9. Model for parasite growth inhibition by SE47'-specific IgG. PVM, parasitophorous vacuole membrane.

Agglutinated schizonts were observed only when Percoll-purified schizonts were cultivated with SE47'-specific IgG. In contrast, agglutinated schizonts were rarely found in the parasite cultures with a 2 to 5% hematocrit. Under standard culture conditions, where parasitemia is usually less than 5%, it is less probable for schizonts to contact and agglutinate with each other. The parasite growth inhibition by SE47'-specific IgG would therefore predominantly result from cross-linking of merozoites in rupturing schizonts. We have no substantial evidence to explain why excess antibody does not lead to complete growth inhibition. It is possible that if a single merozoite fully bound with SE47'-specific IgG still retains invasion capacity, then complete growth inhibition may not be achieved with excess antibodies. Although our data showed that SE47'-specific IgG efficiently inhibited the production of free merozoites, further mechanism studies are required to determine whether additional mechanisms are responsible for the observed growth inhibition, for example, whether antibody to SERA blocks merozoite invasion.

In this study, we present evidence that SE47'-specific IgG

agglutinated merozoites. In addition, SERA protein was also identified on the surface of single merozoites (Fig. 7). However, we could not detect SERA on the merozoite surface when filter-purified merozoites were subsequently washed by low-speed centrifugation (data not shown). It is, therefore, likely that SERA weakly associates with the merozoite, unlike MSP-1, which is covalently bound to the merozoite membrane by GPI anchor. SERA has no predicted membrane-spanning domain or GPI anchor (5).

Miller et al. showed that the merozoites of *Plasmodium knowlesi* agglutinate as they are released or agglutinate within the RBC ghost in the presence of rhesus monkey immune serum (21). Lyon et al. reported that when *P. falciparum* erythrocytic schizonts are incubated with growth-inhibitory immune human serum, antibodies prevent dispersal of merozoites and result in inhibition of parasite growth by formation of immune complexes of merozoites (ICM) (19). The agglutinated merozoites observed in this study are morphologically similar to the structure of reported ICM. Since SERA protein was identified as one of the antigens present in ICM (20), anti-SERA antibodies may contribute to formation of ICM. Investigation of human anti-SERA IgG in areas of endemicity would provide further insights into SERA vaccine development.

Understanding the effector mechanisms that prevent parasite proliferation is highly important for the development of an effective malaria vaccine. The present study provides a molecular basis for a mechanism of parasite growth inhibition by mouse anti-SERA N-terminal domain IgG that primarily prevents merozoite dispersal. In addition, anti-SERA N-terminal-domain IgG-bound parasite cells are killed by complement as previously reported (24). It is therefore of great importance for SERA-based malaria vaccine development to design methods to induce anti-SERA antibodies that effectively cross-link merozoites.

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