the bound antibody was then eluted with 0.1*M* glycine HCl buffer (*p*H 2.8). Isolated rabbit Ab3 447 bound the same antigen that was precipitated by Ab1 (Fig. 2, A and B).

Kennedy *et al.* (*13*) have reported inhibition of a virus-induced tumor by anti-Id directed against an idiotope of a single MAb to SV40 T antigen; this idiotope was not expressed by other MAb's reacting with T antigen. In addition, the modulation of the immune response to tumors after administration of anti-Id has been observed (*14–16*). Our conclusion that Ab2 may carry the internal image of the GA733 tumor-associated antigen is based on the following observations: (i) Ab2 induced the formation of antigen-specific Ab3 in the absence of exposure to antigen in two different species of animals; (ii) Ab2 inhibited binding of Ab1 to target cells; and (iii) the binding of Ab1 to Ab2 was inhibited by tumor antigen. We have also shown that Ab3 CE5 mediated cytolytic effects on human tumor cells in culture. Once IgG2a-producing isotypic switch-variant hybridoma cells are isolated from the IgG1-producing Ab3 CE5 hybridoma cells (*17*), it will be possible to demonstrate tumoricidal activity of Ab3 CE5 in nude mice containing human tumor xenografts (*9*). The beneficial role of anti-Id in cancer patients can then be directly investigated by clinical studies of patients that have been immunized with anti-Id to induce humoral and/or cellular immune responses.

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Perturbation of Red Cell Membrane Structure During Intracellular Maturation of *Plasmodium falciparum*

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An experimental approach, which in this study was applied to the malarial system, can be used to analyze the molecular structure and organization of individual phospholipids in a wide variety of biological membranes. Electron spin resonance spectroscopy was used to investigate the structural modifications of the major red cell phospholipids that occur in erythrocyte membranes infected with the human malarial parasite, *Plasmodium falciparum*. These modifications were correlated with the intracellular developmental stage of the parasite. Phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine were increasingly disordered (fluidized) as infection progressed. This disordering occurred at different rates and to varying extents.

THE PATHOPHYSIOLOGIC MANIFESTATIONS of malaria requires invasion of a host red cell and an obligatory intraerythrocytic developmental process. Invasion of the red cell by *Plasmodium falciparum* has been characterized at the ultrastructural level (*1*). The molecular components of the receptor-mediated attachment step are currently being investigated, with major advances reported by Perkins (*2*) and Ginsburg (*3*). However, little is known about the energetics or molecular mechanisms of red cell penetration by the parasite or about the events that occur in the red cell membrane after parasitic invasion. We propose that key events in the invasion and postinvasion processes involve nonuniform alterations in the structure and organization of the major red cell phospholipids. Alterations in the content and organization of the protein and lipid components of the erythrocyte appear as early as the internalization process. Membrane areas rich in rhomboidally arranged intramembranous particles are evident at the parasite-erythrocyte junction, and a presumptive protein-free, lipid-rich environment in the invaginated portion of the red cell membrane has been observed (*4*). The asymmetric arrangement of phospholipids in the uninfected erythrocyte membrane, with phosphatidylcholine (PC) and sphingomyelin localized in the outer monolayer and phosphatidylethanolamine (PE) and phosphatidylserine (PS) in the inner monolayer (*5*), is altered after penetration of the red cell by the parasite (*6*). PE and PS are increased and PC is decreased in the outer monolayer of the infected red cell after invasion. Ginsburg (*3*) characterized new permeation pathways in the infected cells, which appear at the trophozoite stage and are selective for anions. Other membrane alterations include a more negative transmembrane potential in the later stages of intraerythrocytic development (*7*) and the synthesis and insertion of new and altered membrane proteins on the surface of the infected cell (*8*).

It has been suggested (*3*) that modifications of membrane lipid-lipid and lipid-protein interactions play an important role in erythrocyte penetration, intracellular maturation, and release of malarial parasites. There have been, however, no detailed physicochemical analyses of the red cell membrane during these processes. In order to probe membrane structure at the molecular level, we incorporated specific phospholipid spin labels into the native red cell membrane and compared the electron spin resonance (ESR) spectra arising from uninfected erythrocytes to the spectra arising from erythrocytes parasitized with *P. falciparum* at specific stages of development. We report the conformational modifications induced in individual phospholipid classes in the membrane of the infected erythrocyte at different developmental stages of the parasite.

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We obtained high quality ESR spectra for control and infected erythrocytes labeled with PC 12, PE 12, PS 12, PA 12, and SA 12 (*9*). These phospholipid probes are chemically similar to the endogenous red cell membrane phospholipids and behave physicochemically like their unlabeled red cell counterparts. They also assume the asymmetric distribution exhibited by normal red cell phospholipids (*10, 11*). Representative spectra are shown in Fig. 1. These spectra are typical of those in which the spin probe resides within the lipid bilayer and not free in solution. Furthermore, the labeled infected cells were lysed with saponin to ensure that the ESR signal arose only from the red cell membrane and not from the intracellular parasite. No spin label was found in the released parasites.

The molecular order parameter S, obtained from the spectra of uninfected red cells labeled with the spin probes PC 12, PE 12, PS 12, PA 12, and SA 12, are summarized in Table 1. The order parameter provides a measure of the motional amplitude of the lipid acyl chains. As a rule, the order parameter decreases with increasing phospholipid chain disordering (*12*). The order parameter for PC 12 (0.610) was markedly larger than that for PE 12 (0.540) or PS 12 (0.533), suggesting a more rigid PC bilayer phase and more fluid (disordered) PE and PS phases in the erythrocyte membrane. Tanaka and Ohnishi (*10*) using ESR, also observed this fluidity gradient in the red cell membrane. PC 12 had an order parameter that was 11.4 ± 0.51 percent higher (more ordered) than that of PE 12 and 12.6 ± 0.50 percent higher than that of PS 12. In contrast, the difference in the order parameters for PE 12 and PS 12 was small (1.3 ± 0.45 percent). PA 12, a negatively charged phospholipid present in trace amounts in the red cell membrane, had an order parameter (0.565) similar to those of the inner monolayer phospholipids (PS and PE). SA 12 gave a lower order parameter (0.462) than the phospholipid probes, because of its smaller size and therefore less restricted motion (*13*).

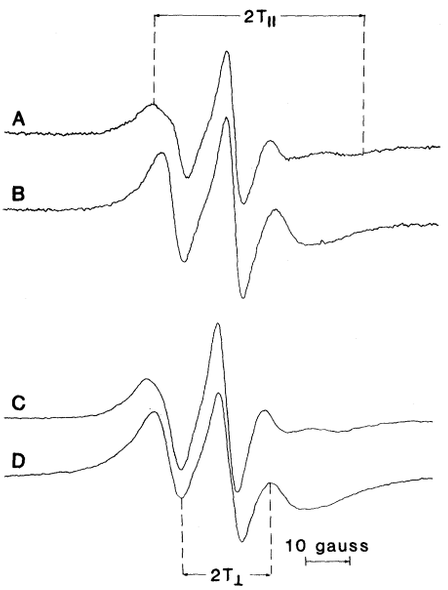

Fig. 1. ESR spectra of uninfected red cells labeled with (A) PC 12 and (C) PS 12 and *P. falciparum*–infected erythrocytes (schizonts) labeled with (B) PC 12 and (D) PS 12. PC 12 was synthesized according to the method of Boss (*20*), and the other spin-labeled lipids were produced by the phospholipase D–mediated headgroup exchange reaction. The parasite used in all studies was *P. falciparum* clone FCR-3/D4 (knob negative) obtained from W. Trager and maintained essentially as described by Trager and Jensen (*21*). Cultures were grown in candle jars in RPMI 1640 supplemented with hypoxanthine (5 μg/ml) and 10% heat-inactivated human A^+ serum and buffered with 20 m*M* Hepes and 1 m*M* $NaHCO_3$. The cultures were diluted every 4 days to 0.5% to 1.0% parasitemia with fresh A^+ erythrocytes. When the parasitemia reached 10% to 15%, the cultures were pooled, centrifuged, and washed with phosphate-buffered saline (PBS) and adjusted to 5% hematocrit with PBS. The method for obtaining a high percentage of stage-specific parasite preparations is a modification of that provided by Aley (*22*). Each preparation was 90% to 95% homogeneous. Erythrocytes were labeled with the spin labels as described in the legend to Fig. 2.

The order parameters obtained from red cells infected with *P. falciparum* are summarized in Table 1. Pronounced alterations in the molecular structure of the red cell phospholipids appear as early as the ring stage. The order parameters obtained from rings labeled with PC 12, PE 12, and PS 12 were decreased compared to those of uninfected controls. PC 12 had an order parameter that was reduced by 7.70 ± 0.19 percent (S = 0.610 to S = 0.563), whereas PE 12 was 4.9 ± 0.09 percent less (S = 0.540 to S = 0.513) and PS 12 was 2.0 ± 0.04 percent less (S = 0.533 to S = 0.522). The order parameter derived from SA 12–labeled rings (S = 0.435) was also decreased (5.6 ± 0.16 percent; S = 0.462 to S = 0.435). The fluidity gradient present in the controls was still evident at the ring stage. However, the differences between PC and PE and PS were attenuated because of the greater disordering of membrane regions occupied by PC (Fig. 2); PS was the least affected at the ring stage.

Further extensive membrane structural disordering (fluidization) was observed at the trophozoite stage (Table 1). The order parameter obtained from PC 12–labeled trophozoites was 6.3 ± 0.15 percent lower (S = 0.563 to S = 0.526) than at the ring stage and 14 ± 0.71 percent less than the control value. PE 12 was further disordered by 5.1 ± 0.16 percent (S = 0.513 to S = 0.484) in the trophozoites, resulting in an order parameter 10.4 ± 0.40 percent less than the control value. PS 12 exhibited a 2.7 ± 0.11 percent decrease (S = 0.522 to S = 0.508) compared to rings and a 4.7 ± 0.21 percent decrease compared to controls. The order parameter for SA 12 was reduced 6.4 ± 0.32 percent further in the trophozoites and 12.0 ± 0.48 percent overall. Thus, at the trophozoite stage, PC and PE were almost twice as disordered as PS, decreasing the difference in order parameter between PC 12 and PS 12 from 7.4 ± 0.22 percent to 3.4 ± 0.09 percent. PS was slightly more ordered than PE in the trophozoite (Fig. 2). This was a dramatic reversal compared to the controls, where the PE environments were more rigid.

Additional membrane disordering was apparent during maturation from the trophozoite to schizont stages. The order parameter calculated for PC 12 was 1.5 ± 0.09 percent less (S = 0.526 to

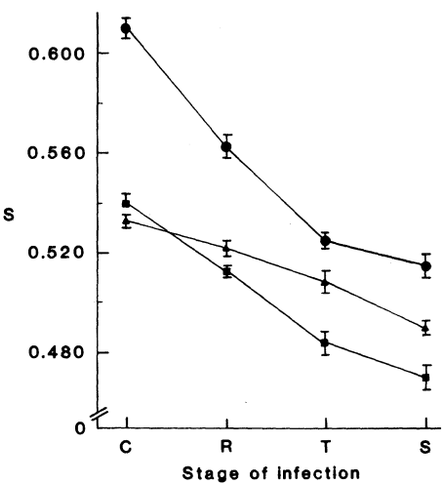

Fig. 2. Order parameter profile of controls (C), rings (R), trophozoites (T), and schizonts (S) labeled with PC 12 (●), PS 12 (▲), and PE 12 (■). Values are the means ± standard deviations. The number of experiments used to determine the order parameters was controls, 6; rings, 4; trophozoites, 4; and schizonts, 4. SA 12 was incorporated into the erythrocyte membrane by mixing 1 volume of control or infected erythrocytes (5×10^8 cells) with 0.5 volume of PBS and 0.5 volume of a bovine serum albumin (BSA)–SA 12 complex for 45 minutes at 37°C. Unincorporated label was removed by washing the erythrocytes with PBS. Red blood cells were labeled with phospholipid spin probes by preparing small sonicated vesicles (250 Å) of the phospholipid spin label and then mixing with either control or infected erythrocytes (5×10^8 cells) at a ratio of 3 mol of membrane lipid to 1 mol of probe for 30 minutes at 37°C. Unincorporated probe was removed by repeated washing. The final ratio of membrane lipid to probe was 120:1 to 150:1. ESR spectra were collected with a spectrometer (Varian E-109) equipped with a cooled nitrogen gas flow system for temperature control. The spectra are the result of one spectral scan (spectral width, 100 G; midfield line, 3250 G; microwave power, 5 mW; modulation amplitude, 1.0 G). All spectra were recorded at 37°C.

Table 1. Means of the molecular order parameters, S, ± standard deviations calculated from the ESR spectra of control and *P. falciparum*–infected red cells labeled with phospholipid spin probes of differing headgroups. The number of experiments used for each determination is given in parentheses. The molecular parameter order was calculated graphically by $S = [(43.7G - 3T_{\perp})/46.1] \times 1.723$, as described in (*13*), and by computer subtraction of the spectra.

Spin label	Control (*6*)	Ring (*4*)	Trophozoite (*4*)	Schizont (*4*)
PC 12	0.610 ± 0.004	0.563 ± 0.005	0.526 ± 0.003	0.515 ± 0.004
PE 12	0.540 ± 0.004	0.513 ± 0.003	0.484 ± 0.004	0.470 ± 0.005
PS 12	0.533 ± 0.003	0.522 ± 0.003	0.508 ± 0.005	0.490 ± 0.003
PA 12	0.565 ± 0.003		0.500 ± 0.004	0.462 ± 0.003
SA 12	0.462 ± 0.003	0.435 ± 0.003	0.406 ± 0.003	0.369 ± 0.003

$S = 0.515$), bringing the total decrease in the order parameter to 15.5 ± 0.62 percent compared to the values for uninfected red cells. The red cell membrane environment occupied by PE 12 also experienced additional disordering (1.6 ± 0.07 percent; $S = 0.484$ to $S = 0.474$) in the schizonts, resulting in a 13 ± 0.62 percent decrease in the order parameter compared to the values for controls. Schizonts labeled with PS 12 had order parameters 3.3 ± 0.64 percent less ($S = 0.508$ to $S = 0.490$) than those of the trophozoites, resulting in an overall decrease in membrane molecular order by 8.0 ± 0.27 percent. SA 12 ($S = 0.406$ to $S = 0.369$) and PA 12 ($S = 0.500$ to $S = 0.462$) also showed considerable further disordering between the trophozoite and schizont stages. The smallest relative amount of molecular disordering experienced by PC and PE occurred between the trophozoite and schizont stages, whereas PS was fluidized most during that period of development (Fig. 2).

It is apparent from these studies that intracellular development of the parasite causes major, nonuniform structural alterations in the red cell phospholipids. The factors responsible for these membrane alterations are not entirely clear at this time. It is unlikely that the decreased molecular order in the infected red cells compared to that in controls is due to age differences between the control and infected erythrocytes. *Plasmodium falciparum* has been reported to show a small preference for invading younger red cells (*14*). Older red cells have been shown by ESR to have more rigid membranes than younger erythrocytes (*15*). Not only were there large decreases in the order parameters between the controls and rings, but also between the rings and trophozoites and between trophozoites and schizonts, demonstrating that nonhomogeneous disordering of red cell phospholipid structure occurred throughout the stages of infection.

The large decrease in molecular order experienced by PC 12 and PE 12 at all stages of infection probably is the result of several factors. After invasion, lipids from the parasite and parasitiphorus vacuole are added to the red cell membrane. The result is that the ratio of cholesterol to phospholipid is decreased (*16*). Such a decrease leads to dilution of the membrane ordering effect caused by cholesterol and thus to decreased order parameters. The parasite lipids themselves could also lead to a less rigid red cell membrane, depending on their chemical and structural properties; information about this is lacking at present. Red cell phospholipids are also likely to be perturbed by association with new surface antigens and proteins inserted into the red cell during parasite maturation (*8*). PS may be perturbed to a lesser extent than PC or PE during infection because of its strong association with the erythrocyte cytoskeleton through its interaction with spectrin (*17*). During infection, the largest relative perturbation of PS occurred between the trophozoite and schizont stages, which precede rupturing of the red cell and release of the infectious merozoites into the circulation.

The phospholipid spin labels used in this investigation are asymmetrically distributed in the red cell membrane. The order parameters obtained for PC 12, which is present in the outer red cell monolayer, are considerably higher than those for PE 12 or PS 12, which are localized in the inner red cell monolayer. These results support the suggestion that a fluidity gradient exists across the erythrocyte bilayer (*10*). Our results suggest that the phospholipids present in the two leaflets of the erythrocyte membrane bilayer experience different degrees of molecular disordering throughout infection. However, the exact distribution of red cell phospholipids in the infected red cells at different stages of infection is not known at this time. Further ESR experiments will help to determine red cell phospholipid asymmetry at different stages of infection.

Kutner *et al.* (*7*) and Ginsburg *et al.* (*3*) reported that new permeation pathways to anionic substances and amino acid derivatives occur in red cells infected with *P. falciparum*. These alterations appeared between the ring and trophozoite stages and persisted during schizogony. It is possible that these alterations in membrane permeability may be related to the pronounced structural modifications we observed in the host red cell membrane matrix during intracellular maturation of the parasites. Having obtained detailed information about the molecular structure of the major red cell phospholipids at different stages of infection, we are now in a position to analyze the relation between the structural organization of each red cell lipid domain and the physiology of the parasite maturation.

The investigative approach outlined in this study can be readily used in the analysis of a wide range of membrane related phenomena such as the deformability of red cells in various disease states—for example, sickle cell anemia, lymphocyte activation, malignant transformation, and the regulation of interactions between membrane skeletal proteins (*18*). The effects of membrane-active drugs (such as tricyclic compounds) on other parasitic diseases, such as leishmaniasis (*19*) can also be investigated with these techniques.

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