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ERYTHROCYTE MEMBRANE ALTERATIONS DUE TO INFECTION WITH *PLASMODIUM BERGHEI*

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SUMMARY

1. The membrane peptide components of erythrocytes from normal mice and animals infected with *Plasmodium berghei* have been analyzed by sodium dodecylsulfate–polyacrylamide electrophoresis.

2. Parasitemia produces significant degradation of the "spectrin" bands of the membranes, as well as of another component with an apparent molecular weight of 112 000. This is associated with the appearance of certain new components which most likely represent split-products rather than parasite constituents.

3. The biochemical changes correlate with major surface deformations demonstrated by scanning electron microscopy.

4. We suggest that normal erythrocyte topology requires the integrity of certain proteins at the inner membrane surface and that the prelytic topologic alterations of the erythrocyte surfaces arise from the degradation of these proteins.

INTRODUCTION

The intraerythrocytic growth of *Plasmodium berghei* alters the host cells' membranes biochemically. Thus membrane cholesterol tends to double and phospholipids quadruple, with concomitant increases in the proportion of phosphatidylethanolamine and a decrease in choline lipids[1,2]. These changes presumably relate to the increased incorporation of free fatty acids and glucose into the phosphatides of erythrocytes infected with the parasites[3,4].

Erythrocyte membranes comprise about 60% protein by mass and we have accordingly explored the possibility that *Pl. berghei* also affects some membrane peptides. We here report that the parasite degrades certain peptides known to lie at the cytoplasmic membrane surface. We also demonstrate the effects of parasitemia upon the topology of the external erythrocyte surface as revealed by scanning electron microscopy.

MATERIALS AND METHODS

We employed 1–2-week-old, 20–40-g white mice (Walter Reed Strain) carried

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at the Department of Medical Zoology of the Walter Reed Army Medical Research Institute, Washington, D.C. In each experiment one group of 6 animals was infected with *Pl. berghei*, strain NY-2 and a parallel uninfected group kept as control. The animals were exsanguinated after 5–7 days, and anticoagulated citrated blood samples fixed with methanol, stained with Giemsa to determine the degree of parasitic infection. About 75% of the erythrocytes from the infected animals contained parasites. Approximately 80% of the infected cells contained one parasite and 20% two or more.

The individual blood samples were packed in ice and transported to Boston by air. The unhemolyzed blood from 2 animals within each group was then combined and stored overnight at 0–4 °C. The erythrocytes were then sedimented at $8000 \times g_{av}$ for 15 min at 20 °C (International Clinical Centrifuge, rotor No. 809). The cells were resuspended in 0.15 M NaCl, 5 mM phosphate, pH 8, and washed 4 times under identical centrifugal conditions. Each sample pair yielded about 1 ml packed erythrocytes. These were then lysed osmotically by addition of 30 ml 5 mM sodium phosphate, pH 8. After 30 min at room temperature the samples were centrifuged at $22\,000 \times g_{av}$ for 30 min at 4 °C (L2-65 B Beckman Centrifuge, rotor No. 50.1), the sediment resuspended in 30 ml of identical buffer, repelleted, and the washing procedure repeated three or four times until no further hemoglobin could be eluted. Infected and control samples were handled identically. Stained membrane preparations gave no evidence for the presence of intact plasmodia.

A limited number of lipid analyses were performed as in Lawrence and Cenedella[1]. We analyzed membrane proteins/peptides by electrophoresis in 1% sodium dodecylsulfate on 5.8% polyacrylamide gels. We used gels 3 mm in diameter, a current of 5 mA/gel, and a temperature of 20 °C[5]. Prior to electrophoresis the erythrocyte membranes were solubilized in 3% sodium dodecyl sulfate at 100 °C for 2 min. Samples of 2 μl containing about 7 μg protein were applied to each gel using Pyronin Y as tracking dye[5]. We employed two methods to make separated membrane peptides visible. The first utilized staining with Coomassie Brilliant Blue as in Fairbanks *et al.*[5]. The second and superior method, that of Weidekamm *et al.*[6], involved formation of covalent fluorescent adducts with membrane proteins, mercaptoethanol, and *o*-phthalaldehyde prior to electrophoresis.

To measure the electrophoretic mobilities of the various bands precisely and to measure their integrated staining intensities, we utilized the Piquant image recognition device as described by Weidekamm *et al.*[7]. This constitutes a computerized flying spot scanner. We employed it in preference to conventional optical scanners because it provides speed, precision, resolution, the ability to evaluate both conventional and fluorescence staining, and because of its unique capacity to simultaneously compute important variables such as peak areas. The system evaluates calibrated photographic negatives of the electrophoresis gels and has a resolution of better than 25 μm. To establish the correlation between relative mobilities of membrane peptides/proteins and their apparent molecular weight, we used a series of standard proteins electrophoresed under identical conditions[5,7]. In all cases membrane peptide/protein bands are numbered as categories from I up, according to decreasing apparent molecular weight[5].

Normal and parasitized cells were prepared for scanning electron microscopy by first washing with Hank's balanced salt solution (Grand Island Biological Com-

pany), 1% in bovine serum albumin (w/v). The cells were then fixed with 2% glutaraldehyde (w/v) in Millonig's buffer pH 7.3[7] washed twice with deionized water and then smeared on chemically clean glass slides. After air drying the specimens were coated at continuously varying angles with gold:palladium (60:40) and viewed with a JSM-U3 scanning electron microscope operated at 25 kV and 0 °C tilting.

RESULTS

Our lipid analyses, although limited to three experiments, support the data of Lawrence and Cenedella[1] and Rao *et al.*[2], in showing a large decrease in membrane choline lipids and an increase in ethanolamine phosphatide.

Very significant changes also occur in the membrane protein of infected cells. Fig. 1 shows the sodium dodecylsulfate–polyacrylamide gel electrophoresis pattern typifying the peptide/protein in the membranes of normal mouse erythrocytes made visible by *o*-phthalaldehyde, and Table I presents the positions and relative staining

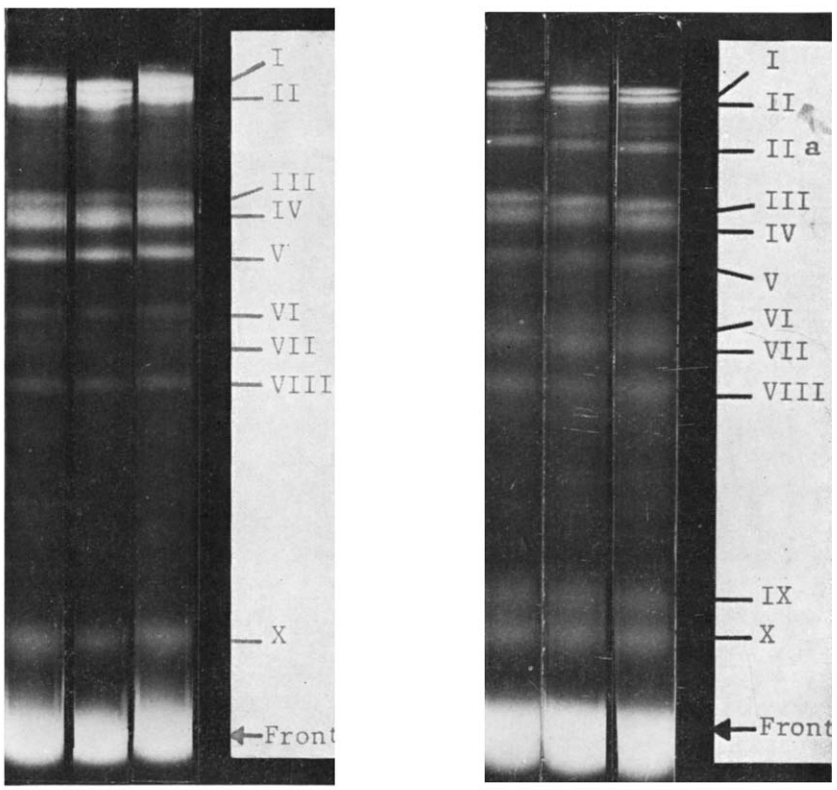

Fig. 1. Sodium dodecylsulfate–polyacrylamide gel electrophoresis of normal mouse erythrocyte membranes. Migration is from top to bottom, 7 μg protein applied per gel and electrophoresed at 5 mA/gel at 20 °C. The peptide components are numbered as specified in text.

Fig. 2. Sodium dodecylsulfate–polyacrylamide gel electrophoresis of erythrocyte membranes derived from mice infected with *Pl. berghei*. Conditions as for Fig. 1.

TABLE I

SODIUM DODECYLSULFATE–POLYACRYLAMIDE GEL ELECTROPHORETIC ANALYSIS OF MEMBRANE PEPTIDES FROM NORMAL AND PARASITIZED ERYTHROCYTES

Peak No.	*Apparent mol. wt***		*Percent of stained area (≈protein concn)*	
	Normal	*Infected*	*Normal*	*Infected*
I	220000	220000	9.1	5.8
II	210000	210000	10.5	9.0
IIa	(168000?)	165000	(2.5)	4.7
III	118000	118000	4.7	5.9
IV	108000	112000	9.3	6.2
V	88000	88000	7.0	6.0
VI	62000	62000	4.0	5.0
VII	54000	54000	3.1	6.9
VIII	44000	44000	4.7	7.5
IX	—	~ 20500	—	8.2
X	16900	16900	10.9	11.6

* Derived from Piquant analysis.
** Based on relative mobilities of standard proteins.

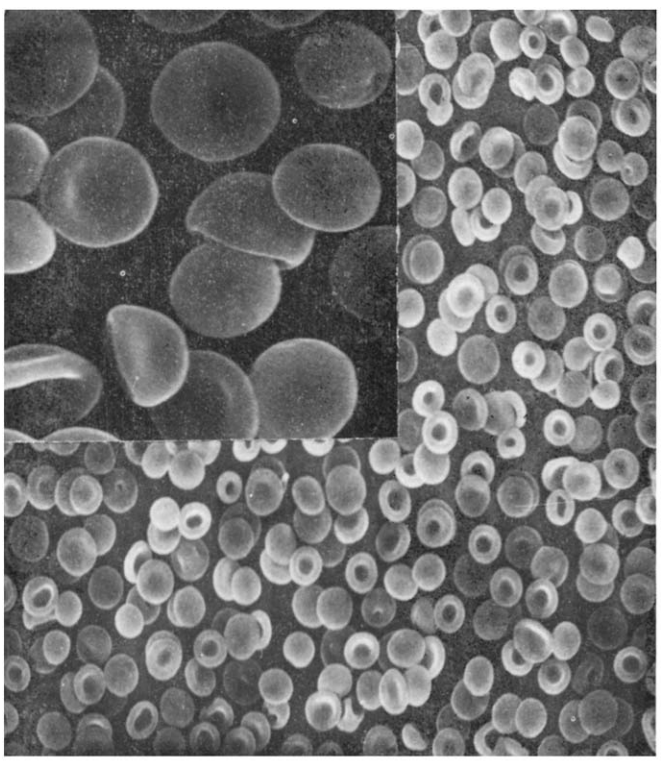

Fig. 3 Scanning electron micrograph of erythrocytes from uninfected mice (magnification 1000 × ; insert magnification 3000 ×).

intensities of the normal major electrophoretic peptide components as determined by the Piquant image recognition device. The electrophoretic pattern is typical of erythrocyte membranes which have been shown to exhibit only slight species differences[9]. Particularly characteristic are the high-molecular-weight Bands I and II (apparent mol. wt about 200000) and a group of 3 bands with apparent molecular weight 88000–118000. Available evidence for all species tested indicates that Components I and II lie at the cytoplasmic membrane surface[10]. The strongly fluorescent band at the front appears associated with membrane lipids primarily[6,7].

Fig. 2 shows the peptide pattern of membranes derived from erythrocytes heavily infected with *Pl. berghei*. The corresponding computer analysis is also given in Table I.

The most significant deviation from the controls is shown in the breakdown of Bands I and II. This correlates with the appearance of a new Band IIa (apparent mol. wt 165000) and an increased intensity of Band VII (apparent mol. wt 54000) and Band VIII (apparent mol. wt 44000). The apparent mol. wts of IIa and VII add to that of I, so that the appearance of IIa and increment in VII could correspond to the cleavage of I into two components of apparent mol. wts of about 165000 and 54000 respectively. We also note a strong decrease in Band IV (apparent mol. wt 112000). The intensification of VII and VIII could thus also partly represent frag-

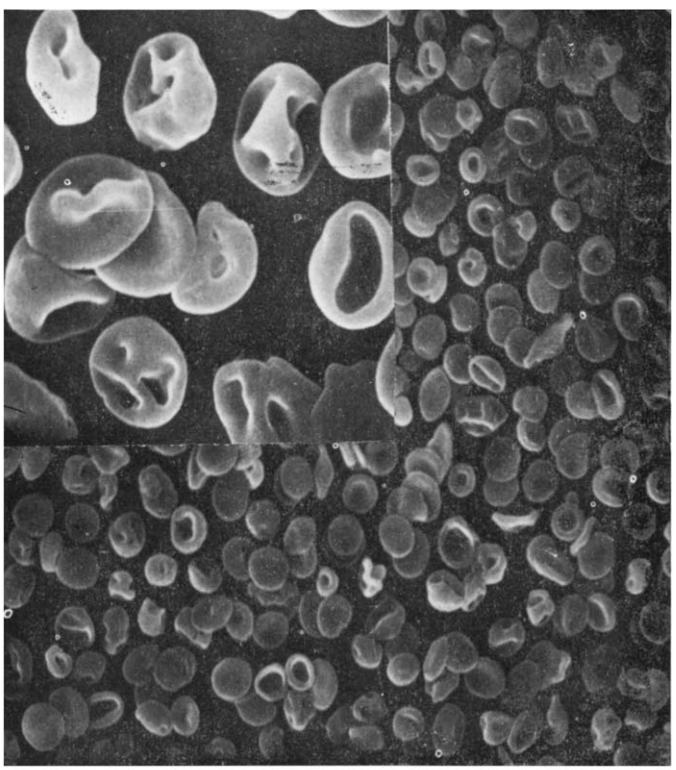

Fig. 4. Scanning electron micrograph of erythrocytes form mice infected with *Pl. berghei*. Magnifications as in Fig. 3.

ments derived from IV. We suspect Band IX to include membrane degradation products, but cannot exclude parasite components; it has no equivalent in uninfected cells. Band X, presumably monomeric hemoglobin, exhibits equivalent intensities and mobilities in control and infected samples.

Scanning electron microscopy (Figs 3 and 4) revealed drastic differences between the surfaces of normal erythocytes (Fig. 3) and those from parasitized blood (Fig. 4). The control cells did not exhibit the biconcave appearance expected of erythrocytes. However, we observed this only in freshly isolated cells which were excluded because of the time required for transport. Nevertheless, out of 500 erythrocytes from parasitized blood, 53 ± 6 (S.D.) % showed the abnormalities of surface topology at $1000\times$ magnification, showed in the insert of Fig. 4 at $3000\times$ magnification.

The deformations observed comprise numerous pleiomorphic anomalies: (1) simple to slit-like surface indentations, (2) severe cup forms, (3) variable and irregular surface protrusions, and (4) single or multiple, round to oval, deep surface indentations. Numerous cells shows more than one defect, and most abnormal cells appeared somewhat smaller than the controls.

DISCUSSION

Several investigators have established that malarial parasitemia alters membrane functions of both normal and parasitized erythrocytes. Thus both exhibit abnormal osmotic fragilities[11], and the rate of hemolysis in malaria exceeds that expected from the number of infected cells. Also Dunn[12] has extended the observations originally published by Overmann[13], that the erythrocytes of monkeys infected with *Pl. knowlesi* exhibit reversible changes of cation permeability. The Na^+ concn in the erythrocytes of diseased monkeys is twice normal, apparently due to impaired active and passive Na^+ fluxes. Intracellular Na^+ concn is also elevated in muscle and liver, but the intracellular K^+ concn is depressed in erythrocytes, unchanged in muscle and elevated in liver[14].

The amino acid transport and incorporation in the red cells of normal and *Pl. knowlesi*-infected *Rhesus* monkeys also differ markedly. Thus McCormick[15] finds the steady state amino concentration of ^{14}C-labelled leucine, isoleucine, cysteine, methionine, and histidine, generally higher in cells from parasitized animals with a proportionately higher incorporation into protein. *Pl. lophurae* also induces altered amino acid transport, but for different amino acids[16]; proline accumulates most, alanine, serine and threonine less so, while methionine, histidine, and leucine were as in the normal cells. Since normal cells were not separated from infected ones in these studies, one cannot be certain what proportion of the observed amino acid transport changes reflects erythrocyte membrane alterations and what is due to the amino acid metabolism of the intracellular parasite.

In any event, the data suggest that malarial parasites effect the production and/or release of some unknown membrane-active agent(s); however, we do not know how such might relate to the infestation and destruction of erythrocytes by the parasites.

As noted earlier intracellular growth of malarial parasites alters the composition and metabolism of erythrocyte membrane lipids[1–4]. Our sodium dodecyl-

sulfate–polyacrylamide gel electrophoresis studies show very clearly that erythrocyte membrane proteins are also altered by parasitic infection, but that not all membrane proteins are affected equally. We observe both the destruction of certain characteristic membrane proteins/peptides as well as the appearance of a few prominent new components. While the degradation of known membrane components can be attributed unambiguously to the action of parasitic proteases or possibly activation of intrinsic membrane proteases, the "new" components must be viewed with caution, since they might possibly represent plasmodial proteins. This is because separation of plasmodial and erythrocyte membranes has met with little success to date and could not be carried out under our conditions of microanalysis.

We doubt that the major "new" electrophoretic components represent significant parasite contamination because (1) no intact parasites were observed in our erythrocyte membrane preparations, and (2) the calculations of Lawrence and Cenedella[1] shows that the lipids of *Pl. berghei* contribute only 5.8% of the total lipids of parasitized erythrocytes. Even if we assume that the parasite membranes contain an improbably high proportion of protein, *e.g.* 80% compared with 60% in erythrocytes, the total contribution of the parasite proteins in sodium dodecylsulfate–polyacrylamide gel electrophoresis should not exceed 10%. In actual fact it should be less since we operated at maximally 75% infected cells. Moreover whatever the contribution is of the total parasite protein, it would probably be distributed among several components, and thus be undetectable.

The decrease in Bands I and II amounts to about 25% together with 40% in Band I assuming no change in staining properties. Since about 25% of the cells were not infected, the degree of breakdown in the diseased cells is presumably closer to 55% for Band I.

The second major site of protein degradation is in the glycoprotein area, specifically in Band IV, which is decreased by about 35%. Again accounting for the proportion of uninfected cells, the degradation in the parasitized cells would approximate 48%.

The appearance of the new Bands IIa and IX, as well as the increments in Bands VII and VIII can be partly explained by assuming that I cleaves into several components, IIa and material migrating with VII and VIII, and that Band IV also splits into components migrating with Bands VII, VIII and IX.

As noted before, we cannot unambiguously eliminate possible contributions from the parasites. However there can be no doubt that *Pl. berghei* degrades certain membrane proteins. Moreover this action appears selective for Components I, II and IV, and we find no evidence for generalized, non-specific proteolysis. Our data suggest that the cleaved proteins are attacked only at limited sites, forming a pattern quite different from that obtained when erythrocyte ghosts are treated with diverse proteases[17]. It would appear that even the susceptible proteins expose only a few vulnerable sites *in vivo*.

The degradation of I, II and IV appears of particular interest in view of the extensive changes of external surface topology revealed by scanning electron microscopy and should be considered in terms of the recent studies of Nicolson *et al.*[10,18]. There can be little doubt now that in most erythrocytes Bands I and II represent protein components lying at the cytoplasmic face of the erythrocyte membrane and also that erythrocytes contain a major glycoprotein, spanning the thickness of the

membrane and migrating in the region of Band IV in most species[19]. The recent data of Nicolson *et al.*[18] show that aggregation of the intracellular proteins represented by I and II specific antibodies caused extensive reorientation of the carbohydrate moieties on the extracellular surface of human erythrocyte membranes in a transmembrane effect, which they relate to a reorientation of the major glycoprotein. We suggest that the breakdown of I and II may lead to a related transmembrane perturbation which we recognize in our scanning electron micrographs. However, we appreciate that the established membrane lipid changes may also severely alter the surface topology of the parasitized cells.

Recently Bodammer and Bahr[20] published scanning electron micrographs, showing "metabolic windows" in the erythrocyte membrane after infection with *Pl. berghei*. Their data closely resemble our morphologic observations.

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