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9. ABSTRACT
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The lipoidal material from these preparations was extracted using chloroform: methanol (1:1) and concentrated under nitrogen. This material was resuspended in chloroform and thin-layer chromatography was used to separate and identify the phospholipids therein. Antigen "A" contained sphingomyelin, phosphatidylcholine, phosphatidylinositol, and phosphatidylethanolamine. Antigen "B", and uninfected mouse erythrocytic material, contained sphingomyelin, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, and possibly phosphatidylinositol. The absence of phosphatidylserine in Antigen "A" its presence in Antigen "B", and in normal mouse material indicates that the protective Antigen "A" is free of host erythrocytic membrane fragments.

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Qualitative Analysis of Phospholipids Isolated from Nonviable Plasmodium Antigen¹

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BECKWITH, RAANA, SCHENKEL, ROBERT H., AND SILVERMAN, PAUL H. 1975. Qualitative analysis of phospholipids isolated from nonviable plasmodium antigen. *Experimental Parasitology* 37, 164-172. *Plasmodium berghei* infected mouse blood, and *Plasmodium knowlesi* infected monkey blood were processed by the French Press to prepare Antigen "A," a parasitic fraction known to impart immunity, and Antigen "B," a byproduct of Antigen "A" production. Normal mouse erythrocyte material was also prepared.

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INDEX DESCRIPTORS *Plasmodium berghei*, *Plasmodium knowlesi*, Membranes, Antigens, Phospholipids, Host erythrocytic contaminants, Thin-layer Chromatography, Immunity.

Present and future attempts to control malaria will most likely involve the use of vaccines. Antigenic preparations, from *Plasmodium berghei* (D'Antonio *et al.* 1970) and *Plasmodium knowlesi* (Schenkel *et al.* 1973), have been shown to be effective vaccines.

The purpose of this research is to analyze the phospholipid content of the plasmodial antigens prepared by Schenkel *et al.* (1973) (Fig. 1), and in so doing to determine the extent of red blood cell membrane contamination in the antigen.

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MATERIALS AND METHODS

Antigen

Antigen "A" from *Plasmodium berghei* and *P. knowlesi* was prepared according to the method of Schenkel *et al.* (1973). The French Pressure Cell and Press was used to disintegrate both infected and normal host red blood cells at 550 psi. The effluent from the press was centrifuged at 1020 g for 10 min, and the supernatant from this recentrifuged at 16,000 g for 15 min. At this point the parasite fraction, in the sediment, was used to prepare Antigen "A." The supernatant containing host red blood cell stroma was centrifuged at 30,000 g for 20 min. The sediment was discarded and

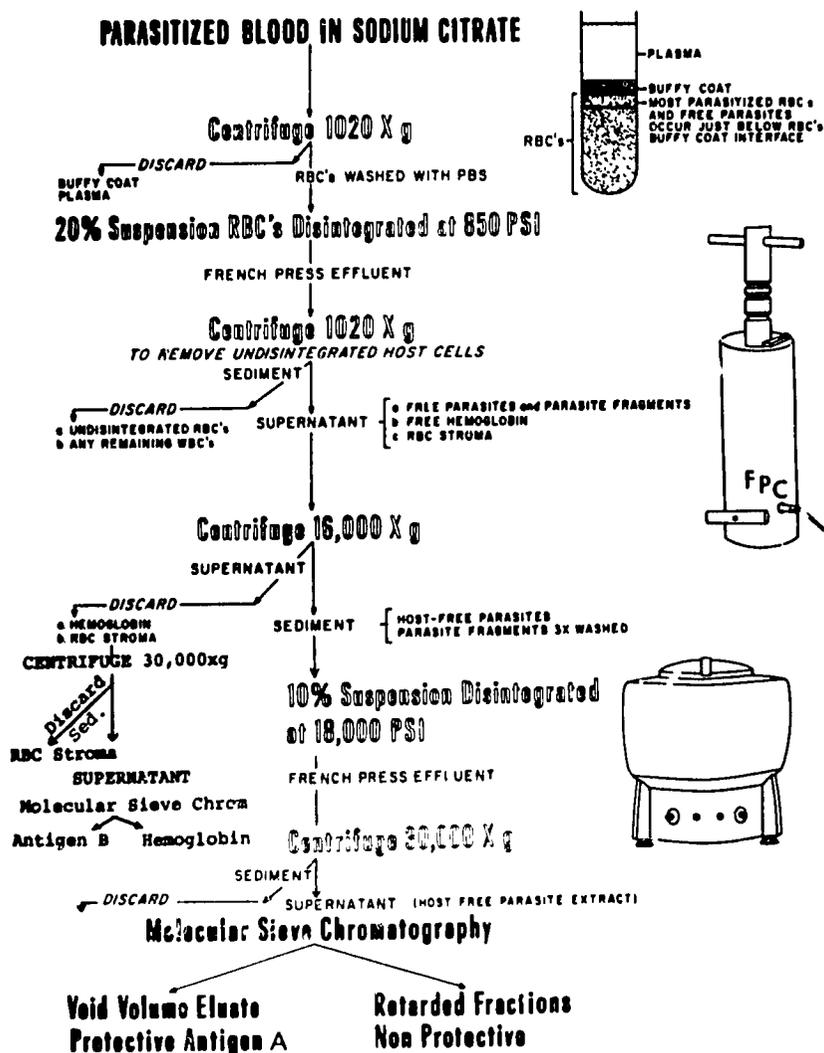


FIG. 1. Antigenic preparation using the French Press method (Schenkel, Simpson and Silverman 1973)

the supernatant was subjected to molecular sieve chromatography to separate Antigen "B" from the hemoglobin fraction, as demonstrated in Fig. 1. Mouse normal red cells (mouse_n) were prepared from uninfected, normal mouse blood in a fashion similar to the preparation of Antigen "B."

Plates

Commercially prepared silica gel TLC plates 20 × 30 cm (without fluorescence indicator) were obtained from EM Laboratories (Elmsford, N. Y.). These were cut

into 10 × 10 cm squares and the origins for spots were marked in pencil. Immediately before each plate was used, it was heated at 110 C for one hour to drive out fixed water (Wien 1960). It was then cooled at room temperature for 30 min, during which time the antigen was processed for lipid analysis.

Antigenic Lipid Extraction

Since no difference in phospholipid content was observed between fresh frozen, or lyophilized antigen, the material was

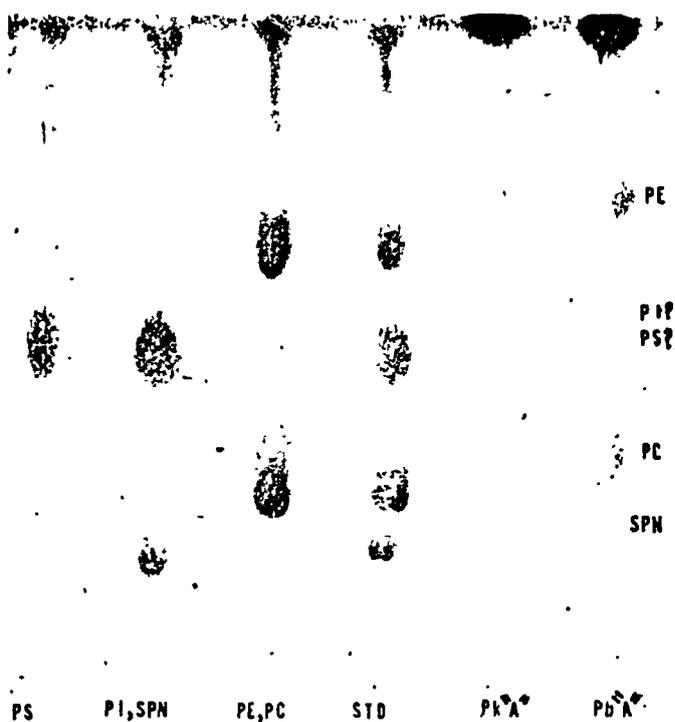


FIG. 2. Pb "A" and Pk "A" are Antigen A prepared from *Plasmodium berghei* and *Plasmodium knowlesi* infected blood, developed in C M A W (25 15 4 2) and visualized with sulfuric acid spray. Abbreviations: PC = phosphatidylcholine, PE = phosphatidylethanolamine, PI = phosphatidylinositol, PS = phosphatidylserine, SPN = sphingomyelin, ? = the presence or absence of the indicated phosphatide, STD = combined standards

used most often in a lyophilized state. The following procedure was carried out at room temperature.

Lyophilized antigen (stored at -20°C) was reconstituted to its original volume using double distilled water. From this material 1.0 ml was centrifuged at 160 g for six minutes. The supernatant was placed in a 5.0 ml screw cap tube and 1.0 ml of methanol was added. The tightly capped tube was then agitated for 10 seconds at setting No. 5 on a Super-Mixer (Matheson Scientific, Chicago, Illinois). To the aqueous-methanol solution was added 1.0 ml of chloroform and this solution was agitated at No. 5 for one minute. The mixture was centrifuged at 200 g for six minutes. Using a disposable pipet, the chloroform layer, containing the lipids, was removed from the resultant biphasic sys-

tem, and transferred to a clean, dry 5-ml beaker. The content of the beaker was taken to dryness under a gentle constant stream of nitrogen

Phospholipid Separation

The lipid material was resuspended in approximately 100 μl of chloroform. Calibrated 5 μl pipets were used to apply the sample as a 15 μl spot (the rest of the chloroform evaporated) on a silica gel plate heated and cooled as previously described. Spots were placed 15 cm apart and 2 cm from the bottom edge. Each 15- μl spot represented the total lipid content from 1.0 ml of antigen.

After spotting, the plates were dried at room temperature for three to five minutes and developed in a glass chromatographic chamber equilibrated with developing sol-

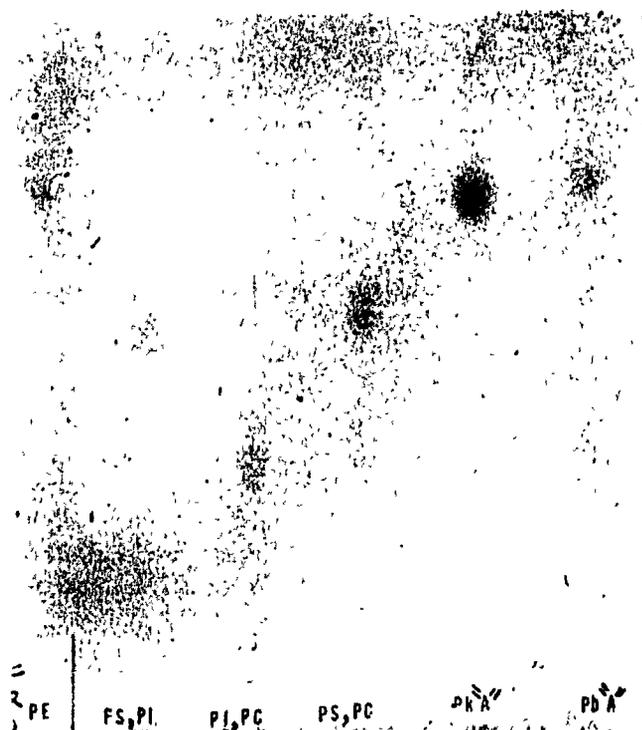


FIG. 3. Pb "A" and Pk "A" are Antigen A prepared from *Plasmodium berghei* and *Plasmodium knowlesi* infected blood, developed in C M A W (25:15:4:2) and visualized with ninhydrin spray. Abbreviations: PC = phosphatidylcholine, PE = phosphatidylethanolamine, PI = phosphatidylinositol, PS = phosphatidylserine.

vent for 30 min immediately prior to use. Phospholipid separation was achieved by one dimensional ascending chromatography. One mobile phase was used per plate. The solvent giving the best result was chloroform-methanol-acetic acid-water (25:15:4:2) (Skiński *et al.* 1964). The solvent front was allowed to migrate 10 cm, whereupon the plate was removed from the chamber and placed on a hot plate for two minutes at 37 C.

Visualization and Identification

A 50% aqueous sulfuric acid spray (Marinetti 1962, Wallace *et al.* 1965) was used due to its high sensitivity to organic substances and its permanency. The plate was sprayed for about 30 sec and then heated in an oven at 110 C for five minutes.

A preparation of ninhydrin in 0.5% butanol (Nutritional Biochemicals, Cleveland, Ohio) was used as a special test for phospholipids with free amino groups like phosphatidylserine and phosphatidylethanolamine. The plate was heated at 70 C for five minutes before and after the spray was used.

Pure samples of phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, and sphingomyelin were obtained from Applied Sciences (State College, Penna) to verify antigenic phospholipid identifications. Ratio of fractions (*r_f*) values were calculated for these pure samples and for those of the antigen phospholipids.

RESULTS

Figures 2 and 4 show that Antigens "A" and "B" are similar in phospholipid con-

tent. But Figs. 3 and 5 demonstrate the absence of phosphatidylserine in Antigen "A" and its presence in Antigen "B." The mouse erythrocytic fraction (mouse_n) is the same in phospholipid content as Antigen "B" as indicated in Figs. 6 and 7.

In summary, Antigen "A" from *P. berghei* and *P. knowlesi* infected blood shows sphingomyelin, phosphatidylcholine, phosphatidylinositol, and phosphatidylethanolamine. Whereas Antigen "B" from the two species, and mouse_n has sphingomyelin, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, and possibly phosphatidylinositol.

DISCUSSION

Killby and Silverman (1969) through EM studies observed that the parasites

freed from erythrocytes by means of the French Press appear to be free of host red blood cell membrane fragments. The results of this research further suggest the lack of red cell membrane contamination in the antigen prepared as described by Schenkel *et al* (1973). Antigen "A" contains no phosphatidylserine, whereas its presence is easily demonstrated in Antigen "B" and in uninfected mouse RBC. Both Rock *et al*. (1971) and Zeeuw *et al* (1973) demonstrated the existence of 10-12% phosphatidylserine in plasmodial infected or uninfected monkey erythrocytic membrane. They also stated that this class of phosphatide is virtually absent in the parasite. The results of this stud. prove the existence of phosphatidylserine in normal mouse red blood cells. Since 97% of all phospholipids are in the cell membrane

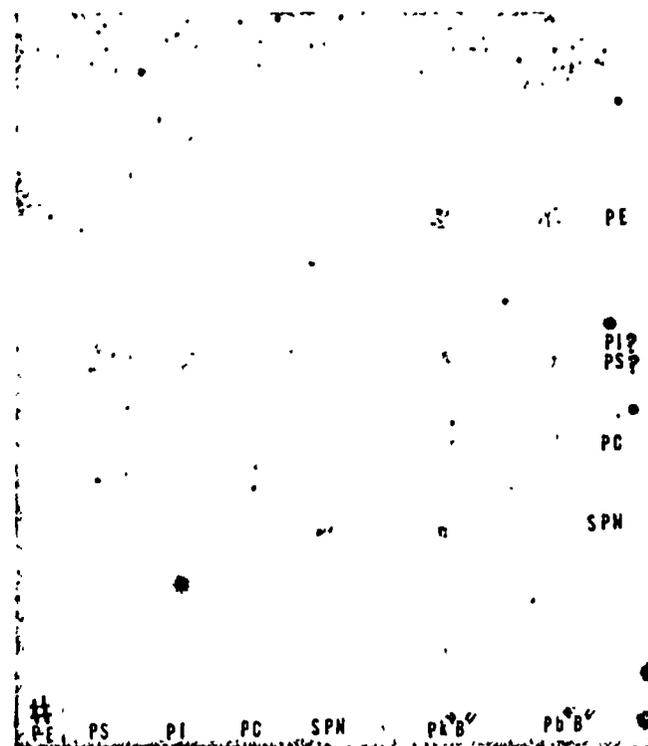


FIG. 4. Pb "B" and Pk "B" are Antigen B prepared from *Plasmodium berghei* and *Plasmodium knowlesi* infected blood, developed in C.M.A.W (25.15.1.2) and visualized with sulfuric acid spray. Abbreviations. PC = phosphatidylcholine, PE = phosphatidylethanolamine, PI = phosphatidylinositol, PS = phosphatidylserine, SPN = sphingomyelin, ? = the presence or absence of the indicated phosphatide.



FIG. 5. Pb "B" and PK "B" are Antigen B prepared from *Plasmodium berghei* and *Plasmodium knoulesi* infected blood, developed in C.M:A:W (25:15:4:2) and visualized with ninhydrin. Abbreviations. PE = phosphatidylethanolamine, PS = phosphatidylserine.

(Van Deenen and de Gier 1964), any contamination of the parasite with host cell membrane would give a ninhydrin positive phosphatidylserine spot. The results indicate the presence of phosphatidylserine in Antigen "B" and its absence in Antigen "A." Thus, it may be concluded that the method utilized by Schenkel *et al.* (1973) for preparing antigenic material from *P. knoulesi* and *P. berghei* produces a parasite antigen free of red blood cell contamination, as assessed by phospholipid content.

It has long been known that phospholipids are responsible for the differential permeability of cells (Wemstein and Marsh 1969) and that they are important regulators of cellular ionic exchange activities as indicated by McElhane and Tourtelotte (1969). According to Ansell and Hawthorne (1964) the existence of phos-

phatidylserine in membranes is related to ion (Na^+ , K^+) transport. This fact would explain the presence of phosphatidylserine in the host erythrocytic membrane (necessary for buffering pH changes from oxidation or reduction of hemoglobin) and its virtual absence in the parasite membrane, since the maturing parasite exists in a fairly stable intracellular environment and it is served by the surrounding host cell membrane (Trager 1974).

Antigen "A" shows a phosphatidylinositol spot approximately the same size and color as the combined spot for phosphatidylserine and possibly phosphatidylinositol of antigen "B." Karlson (1968) states that internal membranes show a higher phosphatidylinositol than external membranes. This might explain why the host erythrocytic fraction (composed of a single plasma membrane) shows little or no phosphatidyl-

inositol and the parasite (with internal membranous structures such as nucleus, vacuoles, ER system) shows a fair amount of this phosphatide.

Lately, it has been realized that phospholipids play an important role in the pathological changes in malaria. Through enzymatic studies, Cenedella *et al* (1968) demonstrated that the parasite hydrolyzes host cell phospholipids, thus increasing the osmotic fragility of blood cells. The severe anemia seen in the late stages of a plasmodial infection has been related to an autoimmune response of the host (McGhee 1964; Kreier *et al* 1966). This autoimmunity may be due to the physiochemical changes in the erythrocytic membrane caused by the metabolism of host phospholipids by the parasite. It has been suggested by Angus *et al.* (1964) and Rao *et*

al. (1969) that the metabolism of host phospholipids release lysophosphatidylcholine and lysophosphatidylethanolamine which have hemolytic properties and may damage uninfected RBC's.

Only very recently it has been realized that phospholipids can act as antigens. Kreier *et al* (1966) and Seed and Kreier (1969) have shown that in malarious animals there occurs an antibody that reacts with phosphatidylcholine and phosphatidylethanolamine. The presence of phospholipids in our protective antigen indicates their possible involvement in the production of immunity against malaria.

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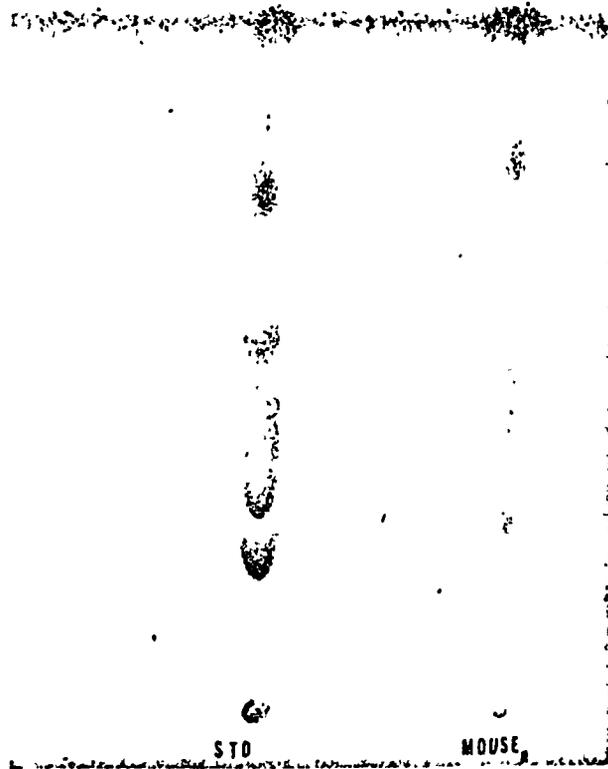


FIG. 6. Mouse_a is material prepared from normal uninfected mouse blood (processed same as Antigen B), developed in C:M:A:W (25:15:4:2) and visualized with sulfuric acid spray. Abbreviations: STD = combined standards.

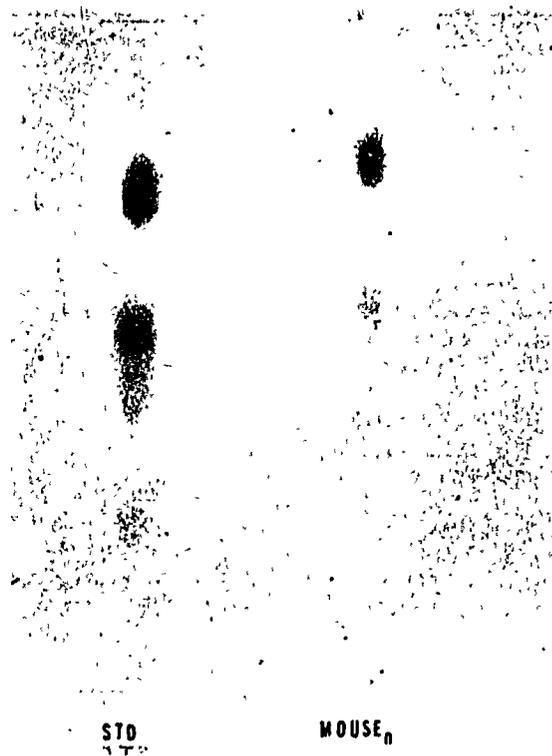


FIG. 7. Mouse_n is material prepared from normal uninfected mouse blood (processed same as Antigen B), developed in C M A W (25.15 4:2) and visualized with ninhydrin. Abbreviations: STD = combined standards.

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