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The Detergent Solubility Properties of a Malarial (Plasmodium knowlesi) Variant Antigen Expressed on the Surface of Infected Erythrocytes

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Four detergents have been compared for identification of the Plasmodium knowlesi variant antigen on infected erythrocytes by immunoprecipitation analysis. Erythrocytes infected with late trophozoite and schizont forms of cloned asexual parasites were labeled by lactoperoxidase-catalyzed radioiodination and extracted either with the anionic detergents sodium dodecyl sulfate (SDS) or cholate, the neutral detergent Triton X-100, or the zwitterion 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS). After addition of Triton X-100 to SDS and cholate extracts, parallel immunoprecipitations of the four extracts were performed using rhesus monkey antisera of defined agglutinability. Identical results were obtained with clone Pk1(A+), which has ^{125}I-variant antigens of M_r 210,000 and 190,000, and with clone Pk1(B+)1+, which has variant antigens of M_r 200,000–205,000. SDS yielded maximal levels of immunoprecipitated ^{125}I-variant antigens. Variant-specific immunoprecipitation was detected in some experiments with Triton X-100 and cholic acid but with significantly lower recovery than with SDS. CHAPS extraction did not yield the variant antigens on immunoprecipitation. The variant antigens could also be identified in Triton X-100-insoluble material by subsequent extraction with SDS, indicating that failure to recover these proteins in the Triton X-100-soluble fraction is due to failure of this detergent to extract the variant antigens rather than to degradation during extraction. We suggest that the ^{125}I-variant antigens either have a structure that renders them intrinsically insoluble in Triton X-100, cholate, or CHAPS, or that they are associated in some way with host cell membrane components that also resist solubilization by these detergents.

Key words: Plasmodium knowlesi, variant antigen, schizont-infected erythrocyte, detergents, radioiodination

Erythrocytes infected with late trophozoite and schizont forms of the simian malaria parasite Plasmodium knowlesi can be specifically agglutinated by antisera

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from infected rhesus monkeys [1]. This agglutination reaction, called the schizont-infected-cell-agglutination (SICA) test [2], thereby identified a new antigen on the surface of infected erythrocytes. Studies with chronically infected animals and with animals infected, drug-cured, and rechallenged with the same isolate showed that this new antigen (SICA-antigen) could be varied by the malaria parasite [2, 3]. It has been suggested that the capacity of P knowlesi to establish chronic infections in rhesus monkeys is due, at least in part, to antigenic variation of the SICA-antigen on schizont-infected erythrocytes [3]. In this way new antigenic variants would evade variant-specific immune responses directed against earlier antigenic phenotypes in a chronic infection or reinfection.

We have recently identified variant antigens of P knowlesi in a strategy involving cloned parasites of different SICA phenotype [4]. Clone Pk1(B+)1+ was produced by antigenic variation in vivo from clone Pk1(A+). These clones have different variant antigen phenotypes as assayed by the SICA test or by indirect immunofluorescence [5]. Since these clones were derived one from the other by induction with agglutinating antibody, it is most likely that these parasites are very similar if not identical phenotypically except for expression of different SICA antigens. Rhesus monkey and rabbit antisera that agglutinate one or other of the clones were prepared and used to immunoprecipitate antigens radiolabeled either by lactoperoxidase-catalyzed radioiodination or by biosynthetic uptake of ^{35}S-methionine [4]. In this way we identified M_r 210,000 and 190,000 antigens of clone Pk1(A+) that were not immunoprecipitated from the other clone by any sera, and only immunoprecipitated from Pk1(A+) by sera that agglutinate shizont-infected cells of this SICA phenotype. Similarly, clone Pk1(B+)1+ was shown to possess antigens of M_r 200,000–205,000 that were only immunoprecipitated from this clone by agglutinating sera, and which were not immunoprecipitated from clone Pk1(A+) by any sera [4]. Since these variant antigens can be radiolabeled by ^{35}S-methionine uptake, we concluded that the P knowlesi variant antigens are parasite proteins rather than altered erythrocyte membrane components.

Our initial report described the methodology for extraction of radioiodinated schizont-infected cells directly with sodium dodecyl sulfate (SDS) followed by immunoprecipitation analysis in the presence of added Triton X-100 [4]. Here we document a comparative study of four different detergents, including SDS and Triton X-100, for their efficacy in extraction and immunoprecipitation of ^{125}I-variant antigens. We also show that the insoluble material after extraction with 1% Triton X-100 can be subsequently extracted with 1% SDS to yield immunoprecipitable ^{125}I-variant antigens. Failure to recover variant antigen from the Triton X-100 extract is therefore not due to proteolysis under these conditions but failure of this detergent to solubilize the variant antigen.

MATERIALS AND METHODS

Cells and Materials

Erythrocytes infected with cloned P knowlesi parasites of different SICA phenotype were obtained from cryopreserved stocks as described previously [4,5]. The derivation of clone Pk1(B+)1+ as an antigenic variant of clone Pk1(A+) is also documented elsewhere [5]. Variant specific agglutinating sera were produced by infection and/or immunization of rhesus monkeys (Macaca mulatta) and by immuni-

zation of rabbits [4,5]. Table I summarizes the specificity and agglutination properties of sera used for immunoprecipitation.

Radioiodination of Schizont-Infected Erythrocytes

Schizont-infected erythrocytes of both clones were purified using step or continuous gradients of Percoll (Pharmacia, Uppsala, Sweden) and radiolabled by lactoperoxidase-catalyzed radioiodination with H_2O_2 supplied directly [6]. Radioiodinated cells were washed in phosphate-buffered saline (PBS: 150 mM NaCl, 20 mM sodium phosphate, pH 7.2) and resuspended at 5×10^8/ml in PBS for extraction with SDS, Triton X-100 or CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate) (Calbiochem-Behring, La Jolla, California) or resuspended at the same cell concentration in HEPES buffer (20 mM HEPES, 150 mM NaCl, pH 8.0) for extraction with cholate. Immediately prior to detergent extraction a cocktail of protease inhibitors [7] was added.

Detergent Extraction

The detergent stock solutions were 2% w/v Triton X-100, 2% w/v SDS or 20 mM CHAPS in PBS, and 1% w/v cholate in HEPES buffer. Equal volumes of these solutions were added with vortexing to aliquots of radioiodinated cell suspension to give a final cell concentration of 2.5×10^8 cells/ml. SDS extraction was performed for 30–40 min at 23°C. Other extractions were for an equal time on ice. Detergent-extracted material was recovered by centrifugation (5 min, Eppendorf Centrifuge) and retention of the supernatant. When the pelleted material from 1% Triton X-100 extraction was subsequently extracted in 1% SDS, it was resuspended with vigorous vortexing in a volume of 1% SDS in PBS equivalent to 2.5×10^8 cells/ml.

Immunoprecipitation

Detergent extracts were used immediately for immunoprecipitation. All samples received an equal volume of 0.15 M NaCl; 5 mM EDTA; 50 mM Tris; 1% w/v

TABLE I. Sera Used for Immunoprecipitation Analysis

	Reciprocal agglutination titer with schizont-infected erythrocytes	
Serum No.[a]	Clone Pk (A+)	Clone Pk (B+)1+
1	< 10	< 10
2	40,960	< 10
3	20,480	< 10
4	10,240	< 10
5	< 10	40,960
6	< 10	40,960
7	< 10	81,920
8	< 10-160	20,480
9	5,120	20,480
10	< 10	< 10
11	< 10	< 10
12	160	< 10

[a]Serum numbers taken from a published table [4], which also describes the derivation of these sera and the number of each donor animal.

Triton X-100, pH 7.4 (NETT); plus 2% w/v bovine serum albumin (BSA). In addition, SDS extracts received 1/10 volume of 20% w/v Triton X-100 in PBS. Volumes of these detergent extracts equivalent to 1.5–2.5 × 10^7 cells were incubated 2 hr at 23°C with 30 μl of each serum to be tested. A 150-μl aliquot of Protein A Sepharose (Pharmacia, Uppsala, Sweden) resuspended 1/1, v/v in NETT plus 1% w/v BSA was added, and the samples were incubated with shaking for 30–60 min at 23°C. Unbound antigen was removed by a series of washes as described previously [6]. Eighty microliters of SDS-sample buffer [8] containing 5% w/v SDS was added to the washed beads and bound material removed within 5-min incubation at 100°C.

Antigen Analysis

Radioiodinated antigens were identified by autoradiography [9] of dried slab gels electrophoresed using the Laemmli buffer system [8]. The gels were 5–15% gradients of acrylamide.

RESULTS

The criterion that we established [4] for identification of the P knowlesi variant antigen was that this molecule should only be immunoprecipitated from detergent extracts of a particular cloned parasite by sera which agglutinate schizont-infected erythrocytes of that clone. Initial experiments using 1% Triton X-100 in an attempt to solubilize and identify the variant antigens were unsuccessful. We therefore made an empirical comparison of the nature of ^{125}I-antigens immunoprecipitated from radioiodinated schizont-infected erythrocytes after extraction with other detergents that might solubilize a different subset of erythrocyte membrane proteins. CHAPS was chosen as a zwitterionic detergent and SDS and cholate were chosen as anionic detergents for comparison with the neutral detergent Triton X-100.

Figure 1 shows that the pattern of immunoprecipitated ^{125}I-proteins for radioiodinated infected cells of clone Pk1(A+) was markedly dependent on the detergent used for antigen extraction. Serum 1 was normal rhesus monkey serum, while all other sera were from monkeys immunized and/or infected with P knowlesi. Comparison of ^{125}I-antigens immunoprecipitated by serum 1 and the other sera indicates that for each detergent several ^{125}I-antigens were specifically immunoprecipitated by antimalarial antibodies. With an SDS-extract of Pk1(A+)-infected cells, two major ^{125}I-antigens of M_r 210,000 and 190,000 together with a minor ^{125}I-antigen of M_r 182,000 fulfilled the specificity criteria of the variant antigens. The accumulated results of several independent experiments [4] showed that the M_r 210,000 and 190,000 ^{125}I-antigens of Pk1(A+) were always immunoprecipitated by sera that agglutinate this clone and never immunoprecipitated by sera that do not agglutinate this clone. Sera such as No. 5, which have high tiers for agglutination of Pk1(B+)1+ (Table I), the clone derived from Pk1(A+) by antigenic variation in vivo, did not immunoprecipitate the M_r 210,000 and M_r 190,000 antigens from Pk1(A+)-infected cells (Fig. 1) [4]. Several other ^{125}I-antigens were immunoprecipitated from SDS-extracts of Pk1(A+), but none of these molecules (M_r 230,000, 165,000, 97,000, and 45,000) were immunoprecipitated only by agglutinating sera. The M_r 97,000 and 45,000 species that are prominent members of this class of antigens comigrate with surface proteins of uninfected rhesus monkey erythrocytes that are labeled by the

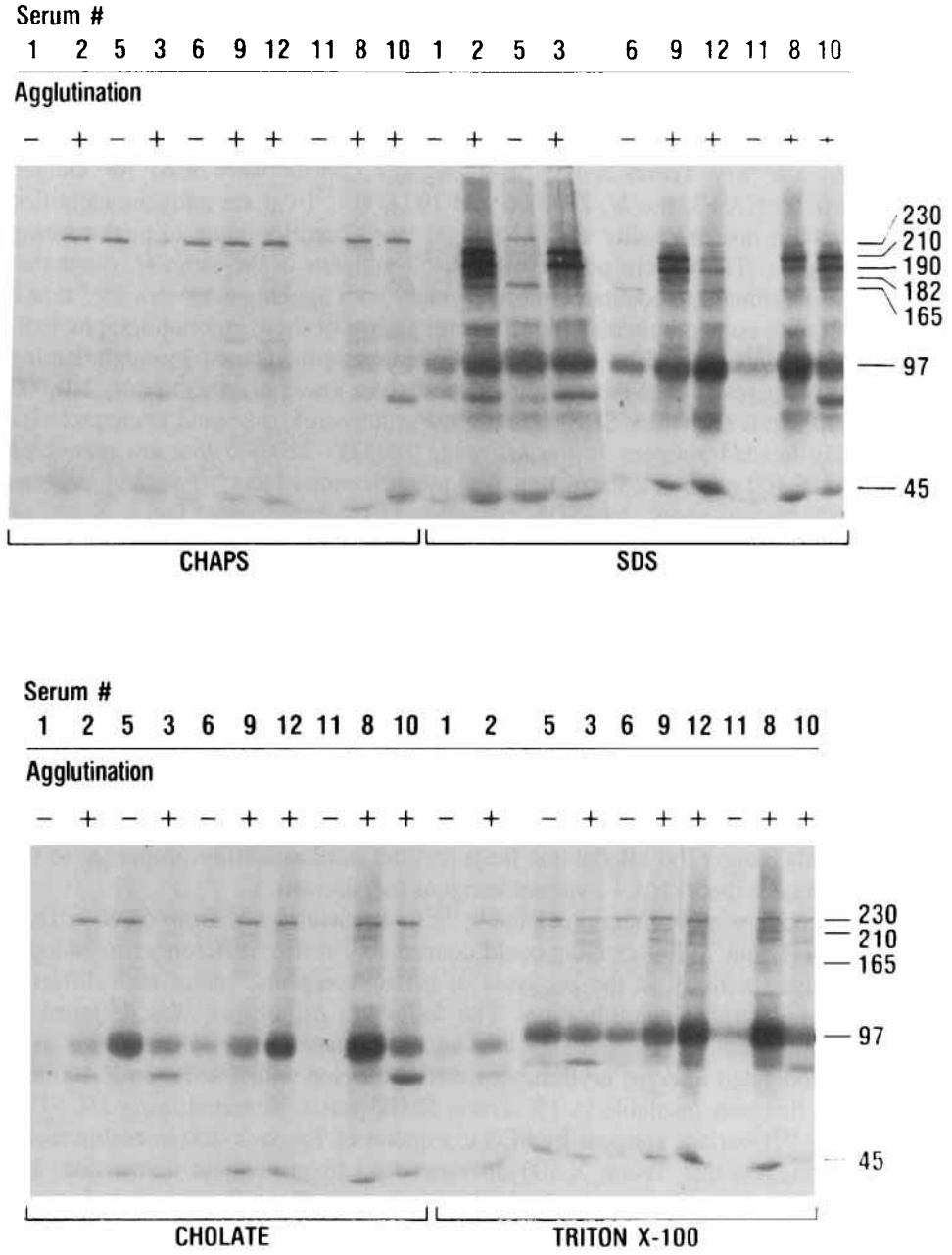


Fig. 1. Comparison of different detergents for identification of radioiodinated variant antigens of clone Pk1(A+) by immunoprecipitation analysis. Labeled schizont-infected erythrocytes were extracted with 10 mM CHAPS, 1% w/v SDS, 0.5% w/v cholate, or 1% w/v Triton X-100, and immunoprecipitation was performed using a panel of sera of defined agglutination specificity (Table 1). ^{125}I-antigens were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The serum used for each gel lane is identified by number (Table I) and its capacity to agglutinate Pk1(A+)-infected cells. +, agglutination; −, no agglutination. M_r or major ^{125}I-proteins shown in kilodaltons. The results of SDS extraction have been published previously [4].

lactoperoxidase method [10]. It is possible that these ^{125}I-antigens represent modified host erythrocyte membrane proteins produced during malaria infection and recognized by antibodies in sera from infected animals. An alternative trivial explanation is that immunoprecipitation of these membrane antigens represents nonspecific binding the the Protein A Sepharose in the presence of bound antigen-antibody complexes.

When 1% w/v Triton X-100 or 0.5% w/v cholate were used for antigen extraction of Pk1(A+), the M_r 210,000 and 190,000 ^{125}I-variant antigens identified with SDS could not be readily seen in the gel lanes corresponding to agglutinating antisera (Fig. 1). There were poorly labeled ^{125}I-antigens in the same M_r range that appeared to be immunoprecipitated more strongly with agglutinating sera (eg, sera 8 and 9) than with nonagglutinating sera. Overexposure of these autoradiographs indicated that two ^{125}I-antigens were specifically immunoprecipitated by agglutinating sera but also suggested that they might be of slightly lower M_r than the M_r 210,000 and 190,000 bands seen after SDS extraction. Further work is needed to characterize very weakly labeled antigens in the M_r range 180,000–220,000 that are recovered after Triton X-100 or cholate extraction. For identification of the ^{125}I-variant antigens these results clearly show that SDS extraction is preferred over Triton X-100 or cholate due to higher yields of specifically immunoprecipitated radioactivity.

Even after overexposure of autoradiographs of immunoprecipitates from CHAPS extractions of Pk1(A+) we could not detect any radioactive antigens in the M_r range 180,000–220,000. The dominant ^{125}-antigen obtained with CHAPS, M_r 230,000, corresponds to a major new ^{125}I-protein identified by electrophoretic analysis of radiolabeled infected cells [6]. This antigen was immunoprecipitated by nonagglutinating and agglutinating sera and is probably not related to antigenic variation. The recovery of radiolabeled M_r 230,000 antigen with CHAPS was greater than with SDS, in contrast to the recovery of radiolabeled variant antigens (Fig. 1).

Similar experiments with clone Pk1(B+)1+ demonstrated that the ^{125}I-variant antigen of this clone (200 kilodaltons [kd]) had the same solubility properties in the four detergents as the Pk1(A+) variant antigens (not shown).

The recovery of immunoprecipitable ^{125}I-variant antigens from SDS extracts but not from Triton X-100 extracts could conceivably reflect differences in endogenous proteolytic activity in the presence of these detergents, rather than different properties of detergent solubilization. The following experiment was designed to address this question.

Radioiodinated infected erythrocytes were extracted with 1% Triton X-100 and the material that was insoluble in 1% Triton X-100 was reextracted using 1% SDS. Recovery of ^{125}I-variant antigens by SDS extraction of Triton X-100-insoluble material would suggest that Triton X-100 does not lead to proteolytic destruction, but rather that it fails to extract these antigens. Figure 2 shows that this was in fact the case. The ^{125}I-variant antigen of Pk1(B+)1+ (200 kd) could be identified in the pattern of total ^{125}I-proteins solubilized with 5% SDS-sample buffer (Fig. 2A, lane a). It was barely detectable in the Triton X-100 extract (lane b), strongly labeled in the SDS extract (lane c), and also strongly labeled in the SDS extract of Triton X-100 insoluble material (lane d). The same results were obtained with clone Pk1(A+). Lanes g and h (Fig. 2A) show the ^{125}I-proteins of both clones that were recovered by SDS extraction of Triton X-100-insoluble material. This figure also shows that the 210- and 190-kd ^{125}I-proteins of Pk1(A+) are of different mobility to the 205–200-kd protein of Pk1(B+)1+.

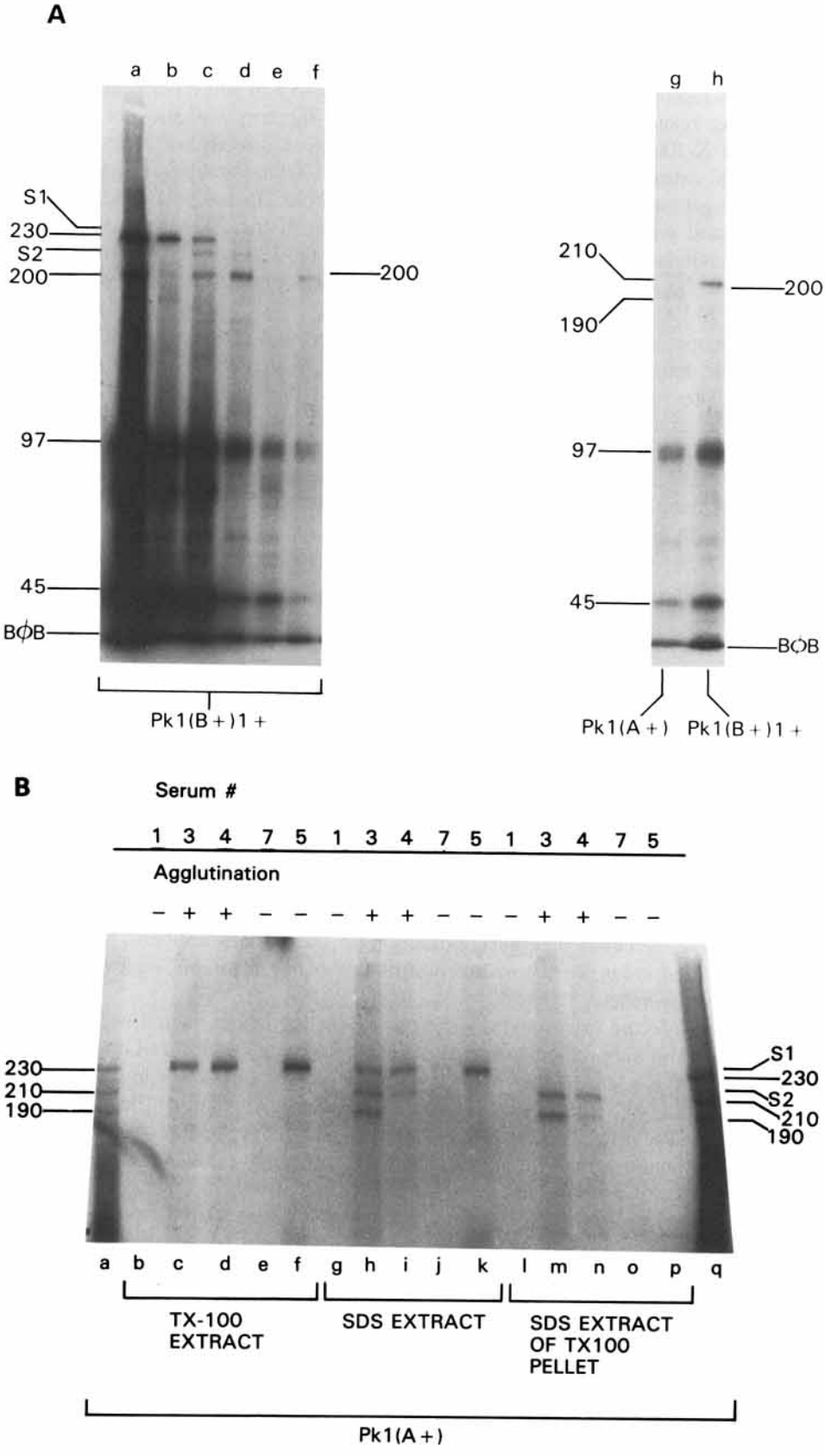
The cytoskeletal protein spectrin (band 1, approximately 240 kd; band 2, approx 215 kd) was radioiodinated in this experiment, indicative of some permeation of the radiolabeling reagents. Note in Figure 2A that ^{125}I-spectrin was also not extracted with Triton X-100, but was extracted with SDS either directly, or after an initial Triton X-100 extraction. Inspection of the pattern of Coomassie blue-stained proteins on the same gel confirmed that the radioiodinated doublet labeled S1 and S2 in Figure 2A comigrated with the spectrin doublet. Extraction of the 200-kd variant protein therefore parallels extraction of spectrin. In contrast, the 230-kd ^{125}I-protein that does not appear to be related to antigenic variation [6] was almost completely extracted with Triton X-100 (compare lanes b and d, Fig. 2A).

Immunoprecipitation of the Triton X-100 extracts and SDS extracts of Triton X-100 insoluble material confirmed these observations. Figure 2B shows the results for Pk1(A+). The variant-specific ^{125}I-antigens of Pk1(A+) (210 and 190 kd) were specifically immunoprecipitated from the SDS extract but not from the Triton X-100 extract. The 230-kd antigen was immunoprecipitated from the Triton X-100 extract but not the SDS extract. Thus the ^{125}I-variant antigens appear to be recovered in greater yield from SDS extracts over Triton X-100 extracts due to their more efficient solubilization by SDS.

DISCUSSION

The malaria parasite Plasmodium knowlesi expresses a variant antigen on the outer face of the erythrocyte membrane of cells containing mature asexual parasites. We previously identified this variant antigen as a malarial protein that can be radiolabeled by lactoperoxidase-catalyzed radioiodination or by uptake of ^{35}S-methionine during parasite growth [4]. In this report we have described the properties of these variant antigens with respect to solubilization and immunoprecipitation using different detergents. The implications of a comparative study using different detergents are twofold: first, the detergent conditions required for solubilization of these erythrocyte membrane-associated malarial antigens (ie, SDS extraction) may be applicable to other new antigens identified serologically in the erythrocyte membrane with other malaria parasites including P falciparum malaria of man [5,10–13]. Second, the properties of differential detergent extraction of the variant antigens may tell us something of the nature of the association of this malarial protein with erythrocyte membrane components.

Purified infected erythrocytes were radioiodinated and extracted with different detergents for immunoprecipitation analysis of the detergent solubilized material. The ^{125}I-variant antigens were either not recovered or recovered in very low yield using the neutral detergent Triton X-100, the anionic detergent cholate, or the zwitterionic detergent CHAPS (Fig. 1). Consistently high yields of immunoprecipitated variant antigens were obtained with the more strongly dissociating and denaturing detergent SDS. After extraction of radioiodinated schizont-infected cells in 1% w/v SDS, Triton X-100 was added to create mixed micelles and thereby minimize the potential denaturing effects of SDS on antibody added subsequently. Antigen-antibody complexes were formed in 1.5% w/v Triton X-100 plus 0.5% w/v SDS and purified by adsorption to Protein A Sepharose. All sera that agglutinated infected cells of clone Pk1(A+) immunoprecipitated ^{125}I-antigens of M_r 210,000–190,000 from this clone. In contrast, all sera that agglutinated infected cells of clone Pk1(B+)1+ immunoprecipitated ^{125}I-


A
a b c d e f
S1
230
S2
200
97
45
BΦB
200
Pk1(B+)1+
g h
210
190
200
97
45
BΦB
Pk1(A+)
Pk1(B+)1+
B
Serum #
1 3 4 7 5 1 3 4 7 5 1 3 4 7 5
Agglutination
– + + – – – + + – – – + + – –
230
210
190
S1
230
S2
210
190
a b c d e f g h i j k l m n o p q
TX-100 EXTRACT
SDS EXTRACT
SDS EXTRACT OF TX100 PELLET
Pk1(A+)

antigens of M_r 200,000–205,000 from Pk1(B+)1+. None of these ^{125}I-antigens were immunoprecipitated from either clone by sera that did not agglutinate that clone (Figs. 1, 2B) [4]. The immunoreactivity of these ^{125}I-antigens in a variant-specific manner with agglutinating sera was therefore not destroyed irreversibly by extraction in 1% w/v SDS. We do not know whether the immunoreactivity of these antigens is unaffected by SDS or whether they regain immunoreactivity upon addition of Triton X-100 to the SDS-extract.

The properties of the new M_r 230,000 antigen identified by radioiodination of P knowlesi schizont-infected cells (Fig. 2) [6] can be contrasted with those of the ^{125}I-variant antigens. The M_r 230,000 antigen was almost completely extracted with 1% Triton X-100, unlike the ^{125}I-variant antigens (Fig. 2). The M_r 230,000 antigen was immunoprecipitated by both agglutinating and nonagglutinating antisera (Figs. 1, 2B) [4]. The recovery of radioactivity in this antigen was less after immunoprecipitation from SDS extracts than with cholate, CHAPS, or Triton X-100 extracts (Fig. 1) [7], despite the fact that the amount of this antigen extracted by SDS was much greater than with the other detergents [7]. Exposure to SDS therefore decreases the immunoreactivity of the M_r 230,000 ^{125}I-antigen.

We have shown that the failure to detect the ^{125}I-variant antigens by immunoprecipitation of Triton X-100 extracts reflects the failure of this detergent to solubilize these proteins. The amount of ^{125}I-radioactivity specifically immunoprecipitated by agglutinating serum was identical for ^{125}I-variant antigens extracted directly from labeled cells with 1% SDS and for ^{125}I-variant antigens extracted from material insoluble in 1% Triton X-100 by subsequent 1% SDS extraction (Fig. 2). The M_r of ^{125}I-variant antigens were also identical after these extraction protocols. Endogenous proteolytic activity, which might remain in Triton X-100 solutions despite the presence of a cocktail of protease inhibitors, cannot account for failure to immunoprecipitate the variant antigens from Triton X-100 extracts.

We next consider, in the broadest sense, possible explanations for the differential detergent-solubility properties of the P knowlesi variant antigens. If the variant antigens were peripheral proteins on the external face of the erythrocyte membrane

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Fig. 2. Comparison of radioiodinated proteins A) and immunoprecipitated antigens B) after extraction of radioiodinated infected erythrocytes with 1% w/v Triton X-100 or 1% w/v SDS directly, or with sequential extraction by 1% w/v Triton X-100 then 1% w/v SDS. A) Lanes a–f show results of SDS-polyacrylamide gel electrophoresis and autoradiography with clone Pk1(B+)1+. Lane a, radiolabeled cells solubilized directly in 5% SDS sample buffer; lane b, solubilization in 1% Triton X-100; lane c, solubilization in 1% SDS; lane d, insoluble material from 1% Triton X-100 treatment solubilized in 1% SDS; lanes e and f, insoluble residues after treatments in lanes c and d, respectively, were solubilized in 5% SDS sample buffer. Lanes g and h show the ^{125}I-proteins of clones Pk1(A+) and Pk1(B+)1+ after extraction with 1% Triton X-100 and subsequent solubilization of the insoluble residue in 1% SDS for electrophoresis. All extracts were diluted in excess 5% SDS sample buffer for electrophoresis. B) Radioiodinated infected cells of clone Pk1(A+) were extracted with 1% w/v Triton X-100 (lanes a–f) or with 1% w/v SDS (lanes g–k). Insoluble material from Triton X-100 extraction was also extracted with 1% w/v SDS (lanes l–p). These extracts were immunoprecipitated using a panel of sera of defined agglutinability with Pk1(A+) (designated by serum number as described elsewhere [4]). The capacity of these sera to agglutinate (+) or not (−) is indicated above each gel lane. The migration positions of the ^{125}I-labeled proteins of M_r 230,000, 210,000, and 190,000 are indicated together with the position of the spectrin bands (S1 and S2). Lane q shows the nonimmunoprecipitated ^{125}I-proteins of the radiolabeled cells solubilized directly in 5% w/v SDS-sample buffer.

interacting with exposed lipids, integral membrane proteins, or glycoconjugates on lipid or glycoproteins, we would expect them to be solubilized by detergents such as Triton X-100, CHAPS, or cholate, which extract the bulk of erythrocyte membrane lipids, glycoproteins and most of the integral membrane proteins [14,15]. If the variant antigens were integral membrane proteins buried in the lipid phase and interacting with lipids and integral membrane proteins, the same solubility properties would be expected. The ^{125}I-variant antigens do not exhibit these solubility properties (Figs. 1, 2). Since the variant antigens are not solubilized by Triton X-100, CHAPS, or cholate, they must either be intrinsically insoluble in these detergents, or they must be linked in some way to erythrocyte membrane components that are also insoluble in these detergents. Additional studies are underway to explore whether the variant antigens are associated with erythrocyte membrane cytoskeletal proteins that have very similar detergent-solubility properties [14,15].

Previous studies on the membrane components of malaria-infected erythrocytes employed Triton X-100 or Nonidet P-40 for antigen solubilization [6,16]. The poor solubility of the variant antigen in Triton X-100, described in this report, explains the failure of these studies to identify this antigen. Experiments in progress show that with P falciparum-infected erythrocytes there are also a set of radioiodinated protein antigens, possibly erythrocyte-membrane-associated antigens, that are not solubilized by Triton X-100 but solubilized by SDS with retention of immunoreactivity. Now that the P knowlesi variant antigen has been identified as a malarial protein expressed on the erythrocyte membrane [4] and its detergent-solubility properties described, it will be of interest to determine the way in which it interacts with the host cell membrane, the nature of any erythrocyte membrane components that it associates with, and its topological disposition with respect to the lipid bilayer and cytoskeleton.

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